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15072

Influence of Induced Changes in Blood Plasma Osmotic Activity on Intestinal Absorption.

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Since isotonic salt solutions become hypotonic within a short time after introduction into ileal segments in dogs,[1] it appeared to be of interest to ascertain what might be the effect of altering the osmotic activity of the blood plasma upon the degree of hypotonicity so developed. It is not possible to predict *a priori* whether the gut epithelium will tend to maintain a certain absolute osmotic activity or a certain difference in osmotic activity between blood plasma and intestinal fluid. Experiments designed to answer this question also throw light on the mechanism of absorption of salt and water. Only two experiments of this type have been performed, but they have statistical validity because of the large number of control observations which have already been reported.

Methods. Adjacent ileal segments were prepared in nembutalized dogs, as previously described,[2] and measured volumes of isotonic solutions of equiosmotic proportions of sodium chloride and sodium sulfate were introduced at the times indicated. Immediately and at intervals thereafter, small measured samples were removed for analysis, and the residues removed and their volume and composition determined 50 min after insertion. Osmotic activity,[3] chloride[4] and sulfate[5] concentrations were determined by the methods indicated. In each experiment one ileal segment was studied without altering the animal osmotically, to serve as a control. Immediately thereafter 100 cc of 5% sodium chloride solution was injected intravenously and within a few minutes the

1 Roepke, R. R., and Visscher, M. B., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 500.

2 Ingraham, R. C., and Visscher, M. B., *Am. J. Physiol.*, 1936, **114**, 676.

3 Baldes, E. J., *J. Sc. Instruments*, 1934, **11**, 223.

4 Van Slyke, D. D., *J. Biol. Chem.*, 1923, **58**, 523.

5 Visscher and Smith, *Experimental Physiology*, Philadelphia, 1934.

second ileal segment was filled with fluid and studied. Samples of femoral arterial blood were drawn at appropriate intervals for osmotic activity and chloride concentration determinations.

Results and Discussion. It may be seen in Table I that when the osmotic activity of the blood plasma is increased by intravenous injection of hypertonic salt solution the osmotic activity of salt solutions placed in ileal segments rises to a level greatly above that found before such a change. In these experiments the greatest lowering of osmotic activity in ileal solutions below plasma levels was at about 20 min after introduction. Comparisons will therefore be made at such times. The figures to be given are interpolations obtained from graphs constructed from the data of Table I.

In the experiments 1a and 2a, while plasma

TABLE I.

Osmotic Activity. Composition and Volume Data from Experiments in Which Plasma Osmotic Activity Was Increased by Hypertonic Salt Solution Injection.

			Plasma		Ileal Fluid			
Experiment No.	Time, min.	Volume, cc	O.A.‡ mM/kg H_2O	$[\bar{Cl}]$ mE/L	O.A.‡ mM/kg H_2O	$[\bar{Cl}]$ mE/L	$[\bar{SO}_4]$ mM/L	Remarks
1a	0	42.1*			159.5	78.6	66.0	Upper segment
	10	2.2†			158.8	61.6	75.0	
	21	2.0†			157.1	38.2	86.4	
	29		161.6	99.6				
	36	2.0†			158.7	16.4	102.6	
	51	9.6†			161.2	4.8	120.6	
	56							Inj. 100 cc 5% NaCl
1b	60	48.0*			161.1	80.4	63.6	Lower segment
	68		173.0	126.8				
	71	2.0†			163.5	66.2	67.0	
	84	2.0†			162.7	41.2	83.4	
	98	2.0†			164.5	22.8	99.0	
	105		170.3	120.7				
	110	13.6*			165.0	12.4	108.6	
2a	0	48.0*			159.5	77.8		Upper segment
	10	2.0†			159.5	67.8		
	25	2.0†			156.3	49.4		
	30		158.8	102.0				
	36	2.0†			156.3	32.8		
	50	32.0†			157.4	24.8		
	62							Inj. 100 cc 5% NaCl
2b	66	48.0*			161.7	78.4	60.0	Lower segment
	72		172.6	108.6				
	79	2.0†			168.0	72.6	41.0	
	92	2.0†			167.0	65.6	37.6	
	104	2.0†			165.5	59.6	38.4	
	109		168.4	112.3				
	116	68.0†			164.5	53.8	36.4	

* Inserted.
† Removed.
‡ Osmotic activity expressed in equivalent concentrations of NaCl.

osmotic activities were normal, the differences between plasma and gut osmotic activities at 20 min were —4.4 and —1.6 mM/kg H_2O. In experiments 1b and 2b, after the plasma osmotic activity had been increased, the corresponding differences 20 min after introduction of fluid were —9.0 and —3.2 mM/kg H_2O. Thus in both instances the osmotic pressure difference maintained across the intestinal epithelium was about twice as great after the hypertonic salt injection as before.

Nevertheless, as will be seen from Table I,

the absolute osmotic activity in the gut solution is considerably greater after the hypertonic salt solution injection than before. At 20 min in experiment 1a the value 157.2 is to be compared with 162.8 in experiment 1b. Thus with an increase of about 10 mM/kg H_2O in plasma osmotic activity, the ileal fluid value rose 5.6 mM/kg H_2O. In experiments 2a and 2b the corresponding values are 157.6 and 167.2 mM/kg H_2O at 20 min, the difference being 9.6 mM/kg H_2O. The increase in plasma osmotic activity was 11.2 mM/kg H_2O for the same times. In both instances the increase in plasma osmotic activity was greater than the change in the gut fluid values.

Thus it is evident that the intestinal epithelial mechanism controlling the osmotic activity of the gut fluid is certainly sensitive to changes in plasma osmotic activity. But it seems unlikely that it is absolutely dependent upon it because if it were one would not expect greater differences in osmotic activity between plasma and gut fluid to occur when the former is raised.

The rates of water absorption in relation to osmotic activity differences are of interest. In experiment 1a a slightly larger fraction of the water was absorbed in 50 min than in experiment 1b. This is in spite of the fact that in the latter case the osmotic gradient between gut and blood over the whole period was 2 or more times as great as in the former. In experiment 2a about 10 cc of water were absorbed while in 2b, after hypertonic salt injection the intestinal fluid increased in volume by about 20 cc. The latter occurred in spite of the osmotic gradient of 3.2 mM/kg H_2O which should have moved water in the opposite direction if normal osmosis were the determining process.

In both experiments 1 and 2 the rate of decline in chloride concentration in the intestinal fluid was less after hypertonic salt solution injection than before. The difference in experiment 1 was not great indicating that an increase of 20+ mE/L in plasma chloride does not have a large effect upon the chloride impoverishment mechanism. The plasma chloride increase in experiment 2 was smaller but the effect on chloride impoverishment was greater.

The sulfate concentration figures in experiments 1a and 1b show the usual increase that occurs when there is a net volume decrease in sulfate-chloride solutions[6] resulting from the fact that the absorption of the sulfate ion is small. No analyses for sulfate were made in experiment 2a, but in 2b the decline in sulfate concentration is a result of the volume increase. It may be noted that the measured volume increase accounts for nearly the entire observed decline in sulfate concentration. There is no obvious reason to account for the fact that in one instance hypertonic salt injection by vein was associated with an intestinal volume increase, and in the other was not. It is possible that there was no causal connection whatever because we[7] have observed such volume increases in isotonic sodium sulfate-chloride solutions without hypertonic salt injection or other alteration. It may be noted that in experiment 2b the osmotic activity of the gut solution at the end is not accounted for by the measured electrolyte. Assuming the presence of sodium chloride and sodium sulfate an osmotic activity of only about 100 mM NaCl/kg H_2O are accounted for. Unfortunately other components were not analyzed. It is possible that bicarbonate might make up the difference because under certain circumstances[7,8] there is a large increase in acid-labile carbon dioxide in gut fluids.

These two experiments are reported primarily because they show something which we have never seen in the absence of poisons in over a hundred control studies,[7,8] namely that the osmotic activity in ileal segment fluids, originally isosmotic with the plasma, rises to values unmistakably higher than the original plasma values. The fact that this occurs when the plasma osmotic activity is raised by hypertonic salt solution injections

6 Ingraham, R. C., and Visscher, M. B., *Am. J. Physiol.*, 1938, **121**, 771.

7 Visscher, M. B., and Roepke, R. R., *Am. J. Physiol.*, 1945, **144**, 468.

8 Visscher, M. B., Roepke, R. R., and Lifson, N., *Am. J. Physiol.*, 1945, **144**, 457.

is highly significant. The results of the two experiments, by themselves, might not have statistical significance, but in the light of the large number of other experiments which can serve as legitimate controls we are convinced of the causal connection between the increase in plasma osmotic activity and the increase in gut fluid osmotic activity. The likelihood of the results occurring fortuitously is less than one chance in ten thousand, in other words, practically negligible.

It seems likely that the activity of the intestinal epithelium in the movement of water across it is influenced greatly by the osmotic activity on both the blood and lumen sides. In neither experiment reported did the increased osmotic gradient across the intestinal epithelium increase the net rate of water absorption. In one case, as noted, the hypertonic salt injection was associated with a negative net water absorption. Nevertheless the fact that gut fluid osmotic activities were changed by the procedure proves that the intestinal epithelium does not act independently of blood osmotic activity, unless the salt concentration itself has a specific effect, which seems to us to be unlikely, though possible. Comparable experiments with non-electrolytes are essential to a settlement of this question.

Summary and Conclusions. Experiments are reported in which the volume, osmotic activity, chloride and sulfate contents of plasma and ileal loop segments are measured, before and after raising the plasma osmotic activity by hypertonic sodium chloride solution injection by vein. The osmotic activity of originally isotonic sodium chloride-sodium sulfate solutions placed in the ileum rises above the pre-injection plasma osmotic activity after 100 cc of 5% sodium chloride solution was injected intravenously. In view of the fact that in more than a hundred control experiments with equiosmotic mixtures of sodium chloride and sodium sulfate the gut fluid osmotic activity has never exceeded the plasma osmotic activity except when poisons were present, the results of these experiments are believed to constitute satisfactory proof that the effect observed was due to the hypertonic salt solution injection.

After the elevation of the plasma osmotic activity the gut fluid values did not approach the former as closely as occurred before the elevation was produced. The net water absorption was not increased in proportion to the increase in osmotic gradient. In fact in one case the direction of net movement was reversed in spite of the larger osmotic gradient. These data add to previous evidence that although normal osmotic forces are probably contributing factors in the transport of water across the intestinal epithelium they are not the primary driving forces determining the direction and magnitude of net water transfer.

15073

Effect of Feeding Glucoascorbic Acid to White Rats, Chicks and Guinea Pigs.*

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Woolley and Krampitz[1] showed that cotton rats and mice failed to grow, developed severe diarrhea and showed hemorrhages in various parts of the body when fed a synthetic diet containing 10% glucoascorbic acid. The condition was prevented when plant ma-

* Published with the approval of the Director of the Wisconsin Agricultural Experiment Station.

† Ghosh Traveling Fellow of the University of Calcutta, India.

1 Woolley, D. W., and Krampitz, L. O., *J. Exp. Med.*, 1943, **78**, 333.

terials such as cabbage or dried grass (Cerophyl) were added to the ration but synthetic vitamin C was without effect. In experiments with guinea pigs Woolley[2] observed the same effect of glucoascorbic acid as observed with cotton rats and mice, but in this case the condition was prevented by ascorbic acid. On this basis he viewed the disease in all the animals as an ascorbic acid deficiency.

Since diarrhea is not usually observed in scorbutic guinea pigs and monkeys the loss of body weight observed by Woolley after feeding glucoascorbic acid might have been the consequence of the diarrhea, and not the result of avitaminosis C. It was, therefore, considered desirable to study the effect of feeding glucoascorbic acid to young rats and chicks which like cotton rats and mice do not require an extrinsic source of vitamin C for their growth, and to guinea pigs for whom vitamin C is a dietary essential.

Experiments with rats. Twenty-one day old male rats were divided into 4 groups. The first group was fed a basal diet consisting of sucrose 75 g, casein 18 g, salt mixture[3] 5 g, corn oil 2 g, thiamine 300 γ, riboflavin 500 γ, pyridoxin 400 γ, Ca *d*-pantothenate 1.5 mg, nicotinic acid 5 mg, choline chloride 100 mg, biotin 20 γ, *i*-inositol 50 mg and 2-methyl-1,4-naphthoquinone 200 γ. For the remaining 3 groups the following were added to the basal ration to give the concentration indicated: glucoascorbic acid (10%), ascorbic acid (10%), or a mixture of glucoascorbic acid (10%) plus ascorbic acid (10%), respectively. The animals were weighed every other day and the symptoms noted. All the animals received a concentrate of vitamins A, D, and E in corn oil once a week.

All the animals except those on the basal diet suffered from diarrhea and failed to grow (Fig. 1). The symptoms were most pronounced in the group receiving both glucoascorbic acid and ascorbic acid and least marked in the group receiving ascorbic acid only. In the group receiving glucoascorbic acid plus ascorbic acid one animal was dead on the eighth day and the other 3 were lethargic and cold to touch.

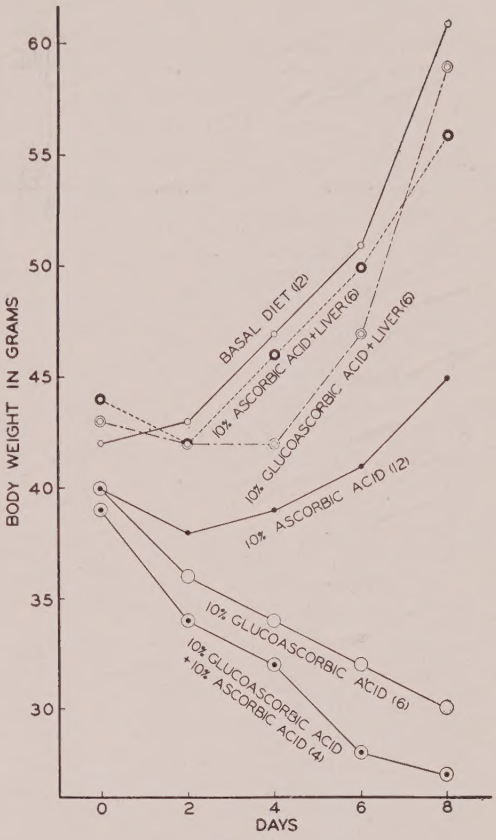

FIG. 1.

Growth curve of rats fed high levels of glucoascorbic acid and ascorbic acid. (Figures in parentheses indicate the number of rats used.)

The surviving rats were killed on the eighth day of the experiment and examined; no hemorrhages or other macroscopic pathological changes were observed in any part of the body. Extracts of the liver and kidneys were prepared by the method of Bessey[4] and titrated with 2,6-dichlorophenol indophenol which was standardized according to method of Menaker and Guerrant.[5] Since glucoascorbic acid also reduces the indophenol dye it is difficult to estimate how much of the reaction depended upon the gluco-

2 Woolley, D. W., *Fed. Proc.*, 1944, **3**, 97.

3 Hegsted, D. M., Mills, R. C., Elvehjem, C. A., and Hart, E. B., *J. Biol. Chem.*, 1941, **138**, 459.

4 Bessey, O. A., *J. Biol. Chem.*, 1938, **126**, 771.

5 Menaker, M. H., and Guerrant, N. B., *Ind. Eng. Chem., Anal. Ed.*, 1938, **10**, 25.

ascorbic acid. It is evident that the total activity in both the liver and kidney was slightly higher in the rat receiving glucoascorbic acid than in those receiving the basal diet alone. When both compounds were added there was a very significant increase in both tissues. Thus, there is no indication of a vitamin C depletion such as occurs in scorbutic guinea pigs.[6,7]

To study the effect of smaller doses of glucoascorbic acid for a longer period of time, groups of four rats were fed glucoascorbic acid mixed with the basal ration at levels of 10, 5, 1 and 0.5%, respectively, for 18 days. As previously observed, the rats receiving 10% glucoascorbic acid failed to gain in weight during the period but when they were transferred to the basal ration they grew as well as normal animals and the diarrhea disappeared. Though the animals receiving the 5% and 1% levels showed diarrhea they grew well. The basal ration produced an average gain of 3.2 g per day. The increases in weight of the other groups during the same period were as follows: 5%, 2.7 g; 1%, 3.7 g; 0.5%, 4.0 g.

Since the animals receiving 10% ascorbic acid also showed diarrhea and failed to grow it was considered that either 10% glucoascorbic acid or 10% ascorbic acid might alter the intestinal flora in such a way that necessary factors for growth were not available. Therefore a group of six 21-day-old rats (male) were fed the basal diet containing 10% glucoascorbic acid plus 2% liver 1:20 powder and another similar group, the basal diet containing 10% ascorbic acid plus 2% liver 1:20 powder. The rats of both the groups had diarrhea but not as severe as that of the groups receiving no liver powder. The animals of both the groups grew almost as well as rats on basal diet alone (Fig. 1). The liver powder did not contain any ascorbic acid but supplied some essential factors which the rats need in the presence of 10% ascorbic acid or glucoascorbic acid.

A group of 4 rats when fed a basal ration containing 10% citric acid grew normally like rats on basal diet alone. This indicated that the action of 10% ascorbic acid or glucoascorbic acid was not directly due to acidity of the rations.

Experiments with chicks. Groups of 4-day-old chicks were placed in electrically heated cages with raised screen bottoms and fed respectively a synthetic ration (486k[8]) containing 2% solubilized liver, the same ration containing 10% ascorbic acid, and the synthetic ration containing 10% glucoascorbic acid, for a period of 15 days. Animals of all the groups grew at equal rates and showed no diarrhea or any other symptoms. Thus, on the basal diet containing 2% solubilized liver no adverse effect of glucoascorbic acid was demonstrable.

Experiments with guinea pigs. Groups of 4 guinea pigs weighing between 190 g and 320 g were fed respectively a ground natural stock ration (Maritime Milling Co. pellets) and the stock ration containing 10% gluco-

TABLE I.
Ascorbic Acid Values of Tissues.

	Liver ascorbic acid (mg/g)	Kidney ascorbic acid (mg/g)	Adrenal ascorbic acid (mg/g)
Rats			
Basal diet	0.32	0.21	
" " + 10% glucoascorbic acid	0.37	0.23	
" " + 10% ascorbic acid	0.39	0.26	
" " + 10% glucoascorbic acid + ascorbic acid	1.12	1.09	
Guinea pigs			
Stock ration	0.07	0.02	0.23
" " + 10% glucoascorbic acid	0.21	0.47	0.40

6 Banerjee, S., *Ann. Biochem. Exp. Med.*, 1943, **3**, 165.

7 Banerjee, S., *Nature*, 1944, **153**, 526.

8 Briggs, G. M., Jr., Luckey, T. D., Mills, R. C., Elvehjem, C. A., and Hart, E. B., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 7.

ascorbic acid. The animals receiving glucoascorbic acid had severe diarrhea from the second day and did not grow like the other group. After 1 week 2 of the animals receiving glucoascorbic acid were fed the same diet which contained in addition 10% ascorbic acid to see if the diarrhea and loss in weight could be counteracted. Two of the guinea pigs receiving glucoascorbic acid diet died on the 9th and 12th days respectively. Two of the guinea pigs receiving 10% ascorbic acid in addition to the glucoascorbic acid diet died on the 14th and 20th day respectively. Ascorbic acid at the level of 10% could not prevent the action of glucoascorbic acid.

To study the possible inhibiting effect of added liver powder on the glucoascorbic acid action observed in experiments with rats, one guinea pig was given a diet consisting of stock ration 84 parts, liver 1:20 powder 6 parts and glucoascorbic acid 10 parts. The animal had no diarrhea and grew well. Unlike rats, the guinea pigs suffered no deleterious effect from the ascorbic acid at the 10% level.

No hemorrhages were observed in any of the guinea pigs which died. The ascorbic acid contents of the liver, kidney and adrenal glands of the guinea pig on glucoascorbic acid were determined and compared with those of one of the animals on stock ration alone. The results are given in Table I. It will be seen that the ascorbic acid activity of the tissue of the glucoascorbic acid fed guinea pigs was very high.

The results of these experiments indicate that the unfavorable effect of glucoascorbic acid in the diet of rats and guinea pigs may not be due entirely to an antivitamin C action as suggested by Woolley but to a lack of some factor other than vitamin C which is normally supplied by the intestinal flora and which is present in the liver 1:20 powder.

Summary. Rats fed a synthetic diet containing 10% glucoascorbic acid lose weight and develop severe diarrhea. Similar but less severe symptoms develop when 10% ascorbic acid is used. Post-mortem examination revealed no hemorrhages in any part of the body. Animals receiving 2% liver powder in addition to either 10% glucoascorbic acid or ascorbic acid grew as well as normal animals although they showed moderate diarrhea. Glucoascorbic acid at levels of 5, 1 and 0.5% had no effect on the growth of rats but the 5 and 1% levels produced diarrhea.

Chicks fed a synthetic ration containing 10% glucoascorbic acid and 2% solubilized liver showed no deleterious effects.

The severe diarrhea and loss of weight in guinea pigs fed a natural diet containing 10% glucoascorbic acid could not be prevented by the addition of 10% ascorbic acid but was prevented by the addition of 6% 1:20 liver powder.

We are indebted to Wallerstein Laboratories, New York, for a liberal supply of glucoascorbic acid; to Merck and Company, Rahway, New Jersey, for the crystalline vitamins; and to Wilson Laboratories, Chicago, Ill., for liver 1:20 powder.

15074 P

Influence of Relaxin on Mammary Development in Sexually Immature Female Rats.*

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The only physiological action so far established for relaxin[1] is the relaxation of the pelvic ligaments in the guinea pig. This preliminary paper reports observations on the effects of relaxin on mammary gland development in immature rats.

Various combinations of estradiol, progesterone and relaxin were given to ovariectomized immature rats to determine the effects on the growth of mammary glands. The estradiol and progesterone were dissolved in sesame oil and injected once a day. The relaxin was dissolved in distilled water and injected in two equal doses, one in the morning and one at night. All injections were made subcutaneously. The animals used were immature female rats which had been ovariectomized at 30 days after birth. After 6 days of postoperative rest they were injected daily with 0.83 μg estradiol for 13 days. This was the standard pre-treatment given all animals before each of the following experiments. A minimum of 4 animals was used in each group cited below and the reactions within each group were uniform.

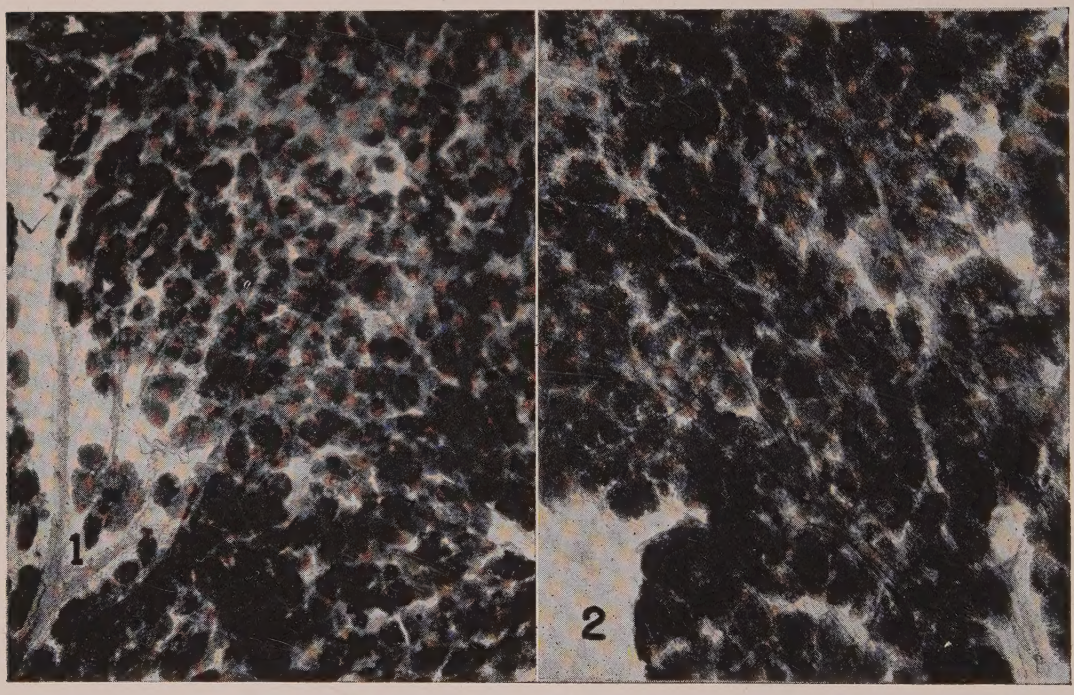

FIG. 1. FIG. 2.

FIG. 1. Mammary gland of female rat ovariectomized at 29 days of age and given 13 days pretreatment with 0.83 μg estradiol daily, followed by daily injections of 0.83 μg estradiol, 1 mg progesterone, and 25 GPU relaxin for 9 days.

FIG. 2. Treatment same as for animal in Fig. 1 but injections of the three hormones were continued through 13 days.

Daily injections of estradiol (0.83 μg per 0.05 cc oil), progesterone (1 mg per 0.05 cc

* Aided by a grant from the Milton Fund to Frederick L. Hisaw.

1 Hisaw, F. L., Zarrow, M. X., Money, W. L., Talmage, R. V. N., and Abramowitz, A. A., *Endocrinology*, 1944, **34**, 122.

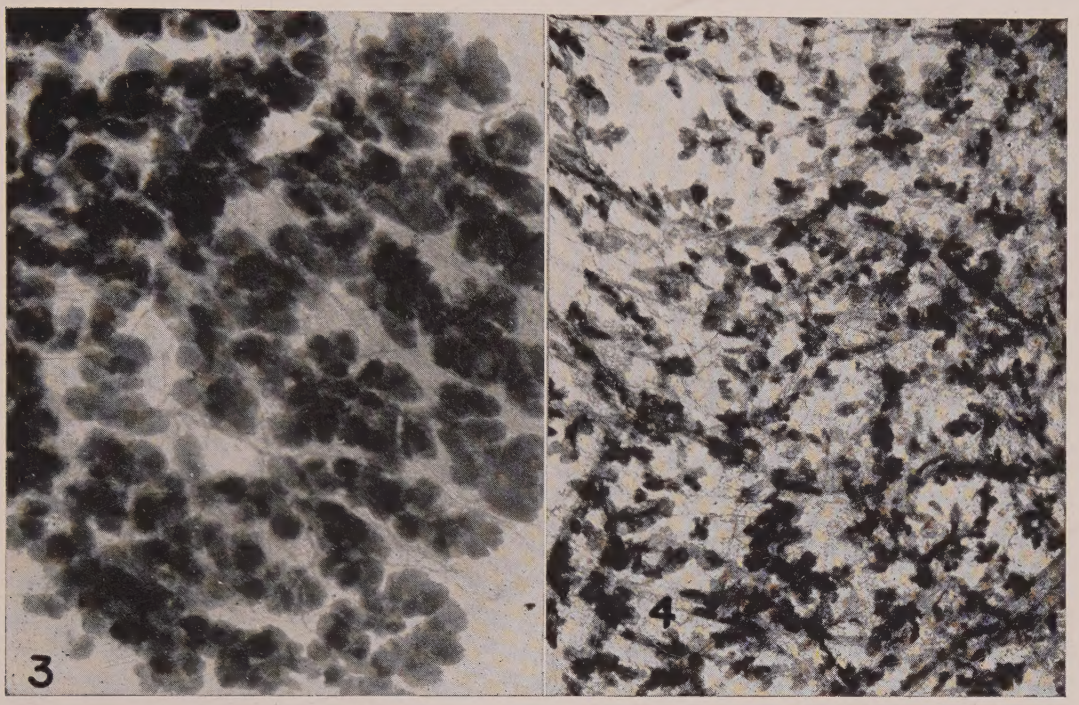

FIG. 3. FIG. 4.

FIG. 3. Mammary gland of a normal pregnant rat on the 18th day of pregnancy.
FIG. 4. Mammary gland of a rat given the same treatment as that of Fig. 1 except relaxin was omitted.

oil), and relaxin (25 guinea pig units[2] per 0.5 cc distilled water) produced growth in the mammary glands by the ninth day of treatment (Fig. 1). By the thirteenth day of this treatment the glands had the appearance of mammary glands during late pregnancy in normal animals (Figs. 2 and 3). Daily injections (for 13 days) of estradiol (0.83 μg) and progesterone (1 mg) but without relaxin caused a slight growth by the ninth day (Fig. 4). This was much less than in animals treated with all 3 hormones (compare Fig. 1 and 4) and there was no increase in the size of the glands following the ninth day. When animals were given estradiol (0.83 μg) and relaxin (25 GPU) or when they were injected with progesterone (1 mg) and relaxin (25 GPU) there was no demonstrable growth in the glands beyond that ordinarily obtained with estradiol or progesterone alone. When the animals were hypophysectomized following the pretreatment and then injected with 0.83 μg estradiol, 1 mg progesterone, and 25 GPU relaxin the mammary glands remained immature.†

Our experiments show that estradiol, progesterone and relaxin given together will cause mammary gland growth and lobulation in immature, ovariectomized female rats. We were unable to obtain mammary gland growth and lobulation with combinations of any two of these hormones and there was no growth of the mammary gland in hypophysectomized rats given all 3 hormones. Therefore we believe that an interaction of relaxin, estradiol and progesterone stimulated mammary gland growth and lobulation in these animals. Hypophysectomy either (1) removed one or more hormones necessary for the interaction to take place or (2) so changed bodily conditions (hormonal or otherwise) that growth of the glands was not obtained.

2 Abramowitz, A. A., Money, W. L., Zarrow, M. X., Talmage, R. V. N., Kleinholz, L. H., and Hisaw, F. L., *Endocrinology*, 1944, **34**, 103.

† Relaxin does not promote growth of the crop glands in pigeons nor maintain luteal function in hypophysectomized rats. Hisaw, unpublished.

15075 P

Heteroplastic Lens Grafts Related to Factors Inhibiting Lens Regeneration in *Triturus.**

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In 18 cases the presumptive lens-forming ectoderm over the optic vesicles of embryos in the early tail-bud stage (similar to Harrison stages 22 to 25) was exchanged between *Amblystoma punctatum* and *Triturus torosus*. The heteroplastic grafts developed normally into large lenses which were observed in a few cases as long as 98 days after operation. At approximately 70 days post-operative, the heteroplastic lenses were excised in some cases, and the larval hosts were killed about 3 weeks later. When the Triturus lens was removed from the Amblystoma eye the result was the same as that which follows lensectomy in the normal Amblystoma eye[1]—a failure of the dorsal rim of the iris to regenerate a lens. When the Amblystoma lens was excised from the Triturus eye, the latter regenerated a lens from the dorsal iris just as it normally does following lensectomy. Therefore, the Amblystoma lens from the early stages of development has the same inhibiting effect on regeneration as does the normal Triturus lens,[2] but its presence for a long time leaves no permanent inhibiting effect upon the Triturus iris.

In 34 cases the lenses of the same two species were exchanged between larvae about 17 mm in length, a period in development not long after the feeding stage. The exchanged lenses survive and grow well for a long time. If they are removed after 70 days the Amblystoma eye again fails to regenerate a lens and the Triturus eye immediately regenerates a lens from the dorsal rim of the iris.

In 14 cases the lenses of young adult *Amblystoma punctatum* eyes were implanted in the lensectomized eyes of adult *Triturus viridescens*. If the Amblystoma lens survives it inhibits regeneration of a lens from the dorsal iris of the Triturus eye. If a cataract condition in the lens becomes extensive it loses its inhibiting capacity even though there may be much normal lens tissue still present. Therefore, throughout life the Amblystoma lens possesses the same factors as the Triturus lens[3] for inhibiting the release of lens regeneration.

In 60 cases the whole or part of the lens of the tadpole eye of *Rana clamitans* was implanted in the lensectomized eye of adult *Triturus viridescens*. In the presence of considerable amount of Rana lens tissue the Triturus eye regenerated a new lens from the dorsal rim of the iris at the same rate which it does after lensectomy.[4] Rana lens therefore does not appear to possess the inhibiting factor.

In 4 cases wax spheres of the same size as the normal lens were placed in lensectomized adult *Triturus viridescens* eyes. They also failed to inhibit lens regeneration. Therefore, mechanical factors associated with lens size do not seem to be important as an inhibiting agent.

* Aided by grants from the John and Mary R. Markle Foundation and the Fluid Research Fund of Yale University.

1 Stone, L. S., and Sapir, P., *J. Exp. Zool.*, 1940, **85**, 71.

2 Dinnean, F. L., *J. Exp. Zool.*, 1942, **90**, 461.

3 Stone, L. S., Proc. Soc. Exp. Biol. and Med., 1943, **54**, 102.

4 Stone, L. S., and Chace, R. R., *Anat. Rec.*, 1941, **79**, 333.

15076 P

A Characteristic of Human Temperature Regulation.*

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The temperature regulatory mechanism maintains rectal temperature of non-basal men sitting at rest in a comfortable environment slightly above 37° C. In certain cold environments which cause shivering, rectal temperature declines to about 35 or 36° C where it tends to become stabilized. This was observed in experiments in which subjects sat immersed to the neck line in well-stirred water baths maintained at 20, 25, or 30° C. (Table I). Rectal temperatures were obtained with thermocouples placed 10 or 15 cm within the rectum. Gastric temperatures taken toward the end of experiments by means of thermocouples inserted in modified Levine tubes were somewhat higher (about 36° C) than rectal temperatures (Table II). Temperatures taken within the stomach are possibly a more reliable index of deep body temperature under the conditions of these experiments than measurements made within the rectum, because the close proximity of surrounding cold water to the junction of the rectal thermocouple may cause falsely low readings.

The fact that deep body temperature became stabilized at approximately the same temperature in all experiments, even though the intensity of shivering necessary to produce sufficient metabolic heat to maintain this temperature varied considerably (Table III), shows that 36° C is a critical temperature at which the thermoregulatory mechanism initiates or attempts to initiate shivering of adequate intensity to prevent further body cooling under these experimental conditions. As might be expected, shivering did not reach its greatest intensity in the experiments until

TABLE I.
Average Rectal Temperatures of Human Subjects During Immersion in Cold Water to the Level of the Neck.

Tw (°C)	No. of subj.	Duration of immersion (min.) 0	15	30	45	60	75	90	105	120	135	150
20	5	37.5	37.2	36.6	36.1	35.8	35.6	35.4	35.4	35.3	—	—
25	4	37.4	37.3	36.9	36.4	36.2	36.0	35.9	35.8	35.6	35.4	35.5
30	4	37.2	37.3	37.0	36.7	36.5	36.3	36.2	36.1	36.1	36.2	36.0

TABLE II.
Comparison of Mean and Extreme Values of Gastric and Rectal Temperatures Existing at the End of the Experiments. All of the Subjects of Table I Who Succeeded in Swallowing the Gastric Thermocouple Are Included.

Tw (°C)	No. of subj.	T rectal (°C)	T gastric (°C)
20	4	35.1 (34.8-35.9)	35.9 (35.2-36.5)
25	3	35.0 (34.2-35.8)	36.2 (36.0-36.5)
30	4	36.0 (35.6-36.8)	36.4 (35.7-37.2)
Mean		35.4 (34.2-36.8)	36.2 (35.2-37.2)

TABLE III.
Average Rate of Metabolic Heat Production (kg-cal/m²/hr) During Immersion in Cold Water. Heat Production Was Calculated from Measurements of the Rate of Oxygen Utilization.

Tw (°C)	No. subj.	Duration of immersion (min.) 0	30	60	90	120	140
20	5	49	174	210	219	—	—
25	4	58	111	138	125	121	140
30	4	53	54	54	58	59	63

* The material in this article should be construed only as the personal opinion of the writer and not as representing the opinion of the Navy Department officially.

body temperature had declined to about this temperature level (Table III). Evidence that 36° C is a temperature in some way important to human thermoregulation has been obtained by others. For example, the results obtained by Burton and Bazett[1] in experiments in which subjects were immersed in relatively warm water showed that metabolic heat production increased above basal level as rectal temperature approached 36° C. DuBois[2] found 36° C to be approximately the lower limit of a series of rectal temperature measurements obtained on a large group of hospital patients. Careful observation under a variety of experimental conditions might elucidate in greater detail the significance to human thermoregulation of this seemingly important deep body temperature level.

1 Burton, A. C., and Bazett, H. C., *Am. J. Physiol.*, 1936, **117**, 36.

2 DuBois, E. F., *Temperature, Its Measurement and Control in Science and Industry*, Rheinhold Publishing Corporation, New York, 1941, p. 24.

15077 P

Tolerance Curve of Vitamin C.*

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The suggestion has been made that intravenous injection of vitamin C, preoperatively, might be of value in preventing the resultant traumatic shock.[1–3] Oral administration has a more prolonged effect upon the blood ascorbic acid concentration and results in greater retention of the vitamin.[4–9] This study was made in an attempt to determine the quantity of synthetic ascorbic acid which, on ingestion, would produce a high concentration of the vitamin in the blood plasma and maintain an increase for a relatively long period of time.

The results of 80 tolerance curves are herein reported. With a lapse of at least one week between each observation, 12 young college women were given 250, 500, 750 and 1000 mg of ascorbic acid, orally. All tests began early in the morning, with omission of breakfast. Lunch was limited, consisting only of vitamin C-free foods. Fasting bloods were taken just before ingestion of the test dose, and subsequent blood samples were drawn each hour thereafter for 6 to 10 hours, depending upon the amount of synthetic vitamin given. Determination of the vitamin C content of the blood plasma was made by the Farmer-Abt micro-method.[10]

The 80 fasting values thus obtained ranged from 0.36 mg to 1.36 mg of ascorbic acid per 100 cc of plasma, the average concentration being 0.79 mg %. During the 3-month period

* This study was made possible through the Dietary Department of the University Hospital and was aided by a grant from the Comly Fund for Research of the Ohio State University.

1 Holmes, H. N., *Science*, 1942, **96**, 384.

2 McDevitt, E., Duryee, A. W., and Lowenstein, B. E., *Southern Med. J.*, 1944, **37**, 208.

3 Ungar, G., *Lancet*, 1943, **1**, 421.

4 Wright, I. S., Lilienfeld, A., and MacLenathen, E., *Arch. Int. Med.*, 1937, **60**, 264.

5 Lozner, E. L., Pohle, F. J., and Taylor, F. H. L., *New England J. Med.*, 1937, **220**, 987.

6 Purinton, H. J., and Schuck, C., *J. Nutrition*, 1943, **26**, 509.

7 Portnoy, B., and Wilkinson, J. F., *Brit. M. J.*, 1938, **1**, 554.

8 Taylor, F. H. L., Chase, D., and Faulkner, J. M., *Biochem. J.*, 1936, **30**, 1119.

9 Farmer, C. J., and Abt, A. F., *J. A. M. A.*, 1938, **111**, 1555.

10 Farmer, C. J., and Abt, A. F., PROC. SOC. EXP. BIOL. AND MED., 1936, **34**, 146.

the fasting values changed very little for any individual. The response to a test dose varied from subject to subject, but neither the amount of increase in plasma ascorbic acid concentration nor its duration were directly related to the concentration of the vitamin in the fasting sample. Tolerance curves of subjects with the lower initial blood plasma levels showed as much rise above fasting as those in which the initial values were higher. In some cases the plasma level remained on a plateau for one to 3 hours after the peak was reached. In other instances the drop from the peak was more pronounced.

The maximum increase in the ascorbic acid concentration occurred one to 4 hours after a test dose was given. After ingestion of 250 mg of vitamin C the plasma levels of most subjects reached their highest point in one or 2 hours. Following the administration of 500, 750, or 1000 mg, peaks were attained, in most instances, in 2 or 3 hours.

A return of the plasma ascorbic acid to approximately the initial level took place, in the majority of cases, 6 hours after 250 mg of the synthetic material was given. Following the administration of 500, 750, or 1000 mg of the vitamin, a slight elevation was maintained for several more hours.

The average increases in the plasma vitamin C content at each hour after the ingestion of 250, 500, 750, and 1000 mg of ascorbic acid are plotted in Fig. 1. Comparison shows that ingestion of 250 mg of synthetic vitamin C did not have as great or as long-lasting an effect as a larger amount. The tolerance curves obtained after the ingestion of 500, 750, and 1000 mg of ascorbic acid are analogous. When these average curves are compared, it is interesting to note that there is less than 0.1 mg variation at any point throughout the 10 hours.

The ingestion of 500 to 1000 mg of synthetic ascorbic acid resulted in similar in-

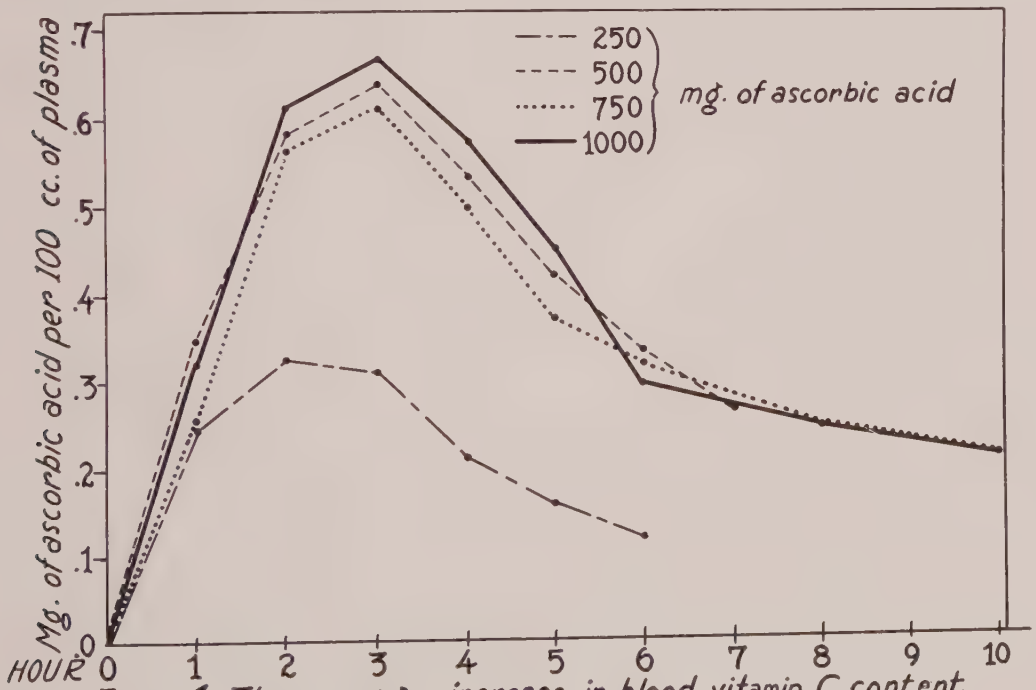

Figure 1 The average increase in blood vitamin C content following ingestion of large amounts of ascorbic acid; 4 curves, each based upon 20 individual observations, the result of giving 250, 500, 750, and 1000 mg. of synthetic ascorbic acid.

creases in the plasma concentration of the vitamin. However, data furnished by concurrent observations[11] on the amount of ascorbic acid *excreted* by the subjects of this study, showed that with the greater intake of the vitamin, more was retained over a 12-hour period.

Sincere appreciation and grateful acknowledgment are due Mrs. Martha Nelson Lewis for assistance and guidance in the organization and completion of this study.

11 Stoughton, M. C., Unpublished data presented for thesis, Ohio State University, 1944.

15078

Effect of Protein Depletion on Plasma Proteins in the Dog Measured by Electrophoretic Analysis.

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Electrophoresis has become a valuable tool for studies of the effect on plasma proteins of disease and other abnormalities in man or experimental animals. Immune sera[1] and sera of patients suffering from diseases such as multiple myelomatosis,[2] pneumonia, peritonitis, acute lymphatic leukemia, etc.[3,4] show electrophoretic patterns differing from the normal. Although most diseases produce a rise in α globulin content of the sera, a rise in β_2 and γ fractions but not in α globulin has been reported in rheumatic fever.[4] In many of these studies the alteration of plasma proteins may have been caused in part by protein depletion as well as by other metabolic shifts attributed to disease. There have been no reports published on the effect of protein depletion alone on the plasma electrophoretic pattern although there is need for such data on the plasma of normal and experimental animals, particularly the dog. In this animal, which has been used extensively in studies on hypoproteinemia and plasma protein regeneration, it has been demonstrated by chemical precipitation methods[5–7] that protein depletion results in a loss of albumin greater than that of the other plasma proteins. In the following are presented the results of studies of the electrophoretic patterns of plasma of dogs before and after depletion, both by protein-free feeding and by plasmapheresis.

Methods. The dogs were depleted of protein by a protein-free diet[8] for 6 to 10 days, after which plasmapheresis was performed for 2 to 5 days. During these days, one-quarter of the blood volume of the dog was removed daily, the cells being returned suspended in saline. This period of plasmapheresis was followed by protein-free feeding alone until the plasma protein concentration was relatively constant at 4.0 to 4.5 g per 100 ml of plasma.

The plasma was analyzed for total nitrogen and non-protein nitrogen by the micro Kjeldahl method. The technic of Howe (1921) as modified by Robinson, Price, and Hogden,[9]

1 Tiselius, A., *Biochem. J.*, 1937, **31**, 1464.

2 Kekwick, R. A., *Biochem. J.*, 1940, **34**, 1248.

3 Longsworth, L. G., Shedlovesky, T., and MacInnes, D. A., *J. Exp. Med.*, 1939, **69**, 399.

4 Luetscher, J. A., Jr., *J. Clin. Invest.*, 1940, **19**, 313.

5 Weech, A. A., Goettsch, E., and Reeves, E. B., *J. Exp. Med.*, 1935, **61**, 299.

6 Holman, R. L., Mahoney, E. B., and Whipple, G. H., *J. Exp. Med.*, 1934, **59**, 251.

7 Seeley, R. D., *Am. J. Physiol.*, in press.

8 Allison, J. B., and Anderson, J. A., *J. Nutrition*, in press.

9 Robinson, H. W., Price, J. W., and Hogden, C. G., *J. Biol. Chem.*, 1937, **120**, 481.

TABLE I.
Composition of Plasma Protein of Dogs Before and After Plasmaphoresis and Protein-Free Feeding.

Dog No.	Wt, kg	Plasma proteins, g/100 ml	Plasma vol., ml	Plasma protein fractions, %				Total circulating plasma proteins, g			Albumin/Globulin		Treatment
					Globulins				Globulins				
				Albumin U = 6.4	α_1 U = 5.2	α_2 U = 4.6	γ U = 1.1	Albumin	α	γ	Howe	Electrophoretic	
28	8.9	5.41	564	44	13	12	8	13.4	7.6	2.4	2.56	0.79	control
	8.8	4.20	460	37	32	—	8	7.1	6.2	1.5	0.86	0.54	depleted
31	5.8	5.96	431	42	6	9	9	10.8	3.9	2.3	1.32	0.72	control
	5.9	3.84	352	27	32	5	9	3.7	5.0	1.2	1.21	0.37	depleted
33	7.9	5.79	443	40	7	8	5	10.2	3.8	1.3	1.60	0.66	control
	7.3	4.32	415	34	15	10	9	6.1	4.5	1.6	1.30	0.52	depleted
52	9.5	6.36	442	29	13	8	12	8.2	5.9	3.4	0.77	0.41	control
	9.0	5.15	388	24	19	9	14	4.8	5.6	2.8	0.60	0.32	depleted
54	12.5	5.35	519	45	16	—	9	12.5	4.4	2.5	1.05	0.82	control
	11.1	4.10	559	21	18	11	11	4.8	6.7	2.5	0.58	0.26	depleted
61	12.3	5.67	566	43	9	11	8	13.8	6.4	2.6	1.26	0.75	control
	11.0	4.40	498	16	20	16	10	3.5	7.9	2.2	0.56	0.19	depleted

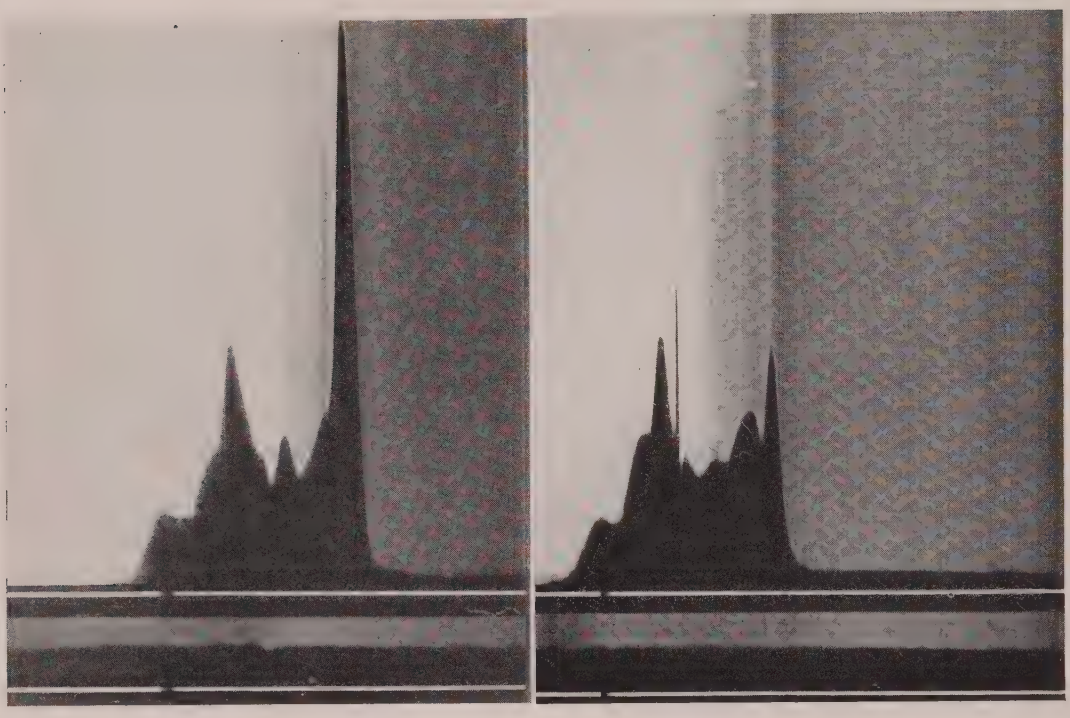

FIG. 1.

Electrophoretic pattern (descending limb) of plasma of normal (1) and protein depleted (2) dogs.

was used for the separation of the plasma proteins into an "albumin" and a "globulin" fraction and for the estimation of the ratio of the 2 fractions. Plasma volume was determined by the method of Gregersen and Stewart.[10]

For electrophoretic analysis,* 10 ml of plasma was diluted with an equal volume of 0.1 N sodium diethylbarbiturate buffer at pH 8.4 ± 0.1. The diluted plasma was dialyzed against 2 liters of the buffer for at least 24 hours in the refrigerator. Electrophoretic analysis was carried out according to the technics of Longsworth,[11] his directions and methods being followed also for the resolution of the scanning patterns. There was no difficulty in separating fractions corresponding to albumin, α globulins, and γ globulin (Fig. 1). The separation of components corresponding to β globulin fractions, however, was not clear cut. Mobility measurement of albumin, α globulins, and γ globulin, showed only slight variations, but considerable variation was found in the proteins of the β globulin group.

Results. In Fig. 1 are shown the descending electrophoretic patterns of the plasma of dog 61 kept on a protein-free diet for 30 days, with plasmaphoresis on the 10th, 11th, and 17th days. The results of electrophoretic analyses of the composition of plasma protein of 6 dogs before and after such depletion are recorded in Table I. Hypoproteinemia occurred in every dog, accompanied by reduction of plasma volume, except in dog 54. The percentage of albumin was reduced, the percentage of α globulins was increased, and the

[10] Gregersen, M. I., and Stewart, J. D., *Am. J. Physiol.*, 1939, **125**, 142.

* The authors wish to express their appreciation for the technical assistance of Miss Shirley Quaid.

[11] Longsworth, L. G., *Chem. Rev.*, 1942, **30**, 323.

[12] Tiselius, A., and Kabat, E. A., *J. Exp. Med.*, 1939, **69**, 119.

γ globulin percentage remained essentially unchanged. The total circulating albumin decreased markedly, the γ globulin tended to decrease slightly, whereas the α globulins changed but little or increased. The maintenance of the circulating α globulins in the depleted state, even though the albumin has been markedly reduced, suggests that the replacement of α globulin in the dog is much more efficient than that of albumin.

The plasma A/G ratios determined by Howe's method were always smaller than those obtained by electrophoresis. Plasma from one normal and one depleted dog was separated into albumin and globulin fractions by Howe's technic. Electrophoretic analyses of these fractions showed that the albumin fraction of the normal dog contained 23% contaminating globulin whereas the globulin fraction contained 6% albumin. The albumin fraction of the depleted dog contained 39% contaminating globulin and the globulin fraction 3% albumin. Thus, incomplete precipitation of the globulins by the Howe method accounted for the higher A/G ratio. The decreased ratios by both methods reflected the relatively greater reduction in the albumin fraction in the depleted state.

Summary and Conclusions. Electrophoretic analyses of plasma showed that protein depletion in dogs resulted in marked decrease in circulating albumin, and slight decrease in γ globulin. In contrast to these decreases the α globulin content remained essentially unchanged or increased. The α globulins, however, always increased in concentration, which was in part due to the accompanying fall of plasma volume. The Howe method for the determination of albumin: globulin ratios resulted in higher ratios than those obtained by electrophoretic analyses, because of the contamination of globulins in the "albumin" fraction.

15079 P

The Antigenicity of *Shigella paradysenteriae* (Flexner) in Saline-in-Mineral-Oil Emulsion.*

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Several reports have recently demonstrated that the immune response to antigens may be markedly prolonged, and often elevated by the suspension of these antigens in the aqueous phase of a saline-in-mineral-oil emulsion.[1–3] Killed acid-fast bacilli have been included also in the oil emulsion in a number of these instances,[3,4] but Freund has demonstrated in rabbits that in some instances at least, this was not a requisite.[2] The Henles have recently obtained extremely favorable results in human subjects injected with influenza virus in saline-in-mineral-oil emulsion.[5]

In the search for a method of improving the response to *Shigella paradysenteriae* vaccines, the oil emulsion technic was investigated in mice, rabbits, and humans. Conditions were chosen for the experimental animals that

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pennsylvania.

1 Freund, J., and McDermott, K., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 548.

2 Freund, J., and Bonanto, M. V., *J. Immunol.*, 1944, **48**, 325.

3 Friedewald, W. F., *J. Exp. Med.*, 1944, **80**, 477.

4 Freund, J., and Walter, A. W., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 47.

5 Henle, W., and Henle, G., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 179.

TABLE I.
Agglutinin Titer in Mice After Receiving 20 μg of *S. paradysenteriæ* Vaccine in Saline, or in Saline-in-oil Emulsion.

Menstruum	Agglutination titer after (weeks)							
	1	2	6	10	12	14	18	22
Saline	40	20	4	4	8	4	5	4
Saline-in-oil emulsion	40	10	16	256	256	256	160	32

could be applied to man. Since it was felt that the inclusion of acid-fast bacilli in the adjuvant mixture would be contra-indicated for human use, because of the danger of tuberculin hypersensitivity reactions,[4] almost all of the observations were made without them.

The oil emulsion vaccines were made essentially according to the technic of Freund.[1] Falba,† a stabilizing agent derived from lanolin, was mixed by electric stirrer with a saline suspension of the alcohol-killed cells of *S. paradysenteriae* until a smooth, pasty emulsion was formed. The mineral oil‡ was then added and stirred in until the emulsion was well dispersed. The proportion of the constituents, antigen in saline: falba: mineral oil was 1:1:4, respectively. All the vaccines were administered subcutaneously. The technics of the agglutination and mouse protective tests are described in detail in a previous publication.[6]

Results in Mice. A series of 35 inbred white Swiss mice were given a single injection of 20 μg (in 0.15 ml) of cells of a strain of *Shigella paradysenteriae* ("V368D")§ in saline. A similar group was injected with the same dose of the identical vaccine suspended in 0.15 ml of oil emulsion. At appropriate intervals, groups of 4-5 mice were exsanguinated from the heart, and the blood pooled. The sera thus obtained were titrated for their antibody content by agglutination test. Table I represents the course of the titers in the two groups over a period of 22 weeks.

As can be seen from these data, the peak titer of the saline menstruum group was reached in one week, and dropped to low levels quite rapidly. The peak titer in the oil emulsion group (about 6 times higher than in the controls) was reached by 10 weeks, and only gradually fell after 14 weeks. It may be noted here that normal mouse serum will practically never agglutinate this strain of *S. paradysenteriae.* It has been shown that peak titers of mouse antiserum for this organism, after injection of large doses of antigen, range from 1:640 to 1:1280. Several other series in mice have confirmed the validity of the above findings.

Three small experiments have also been undertaken in which 2 strains of *Mycobacterium phlei,* and one strain of *M. butyricum*‖ were included in the oil phase of the emulsion vaccine described above. Agglutinin levels determined up to 6 or 8 weeks showed no definitely significant increase ascribable to the presence of the acid-fast bacilli.

Results in rabbits. Groups of 3 rabbits each were injected with 2 doses of *S. paradysenteriae* Flexner "V368D" alcohol killed bacteria 3 days apart (total 50 μg). The antigen was either suspended in saline, in a mineral-oil emulsion, or adsorbed onto alum. The volume of each dose was 0.15 ml for each menstruum. Sample bleedings were taken

† Falba is the trade name of a lanolin-like substance prepared by Pfaltz and Bauer, Inc., New York, N.Y.

‡ The brand of mineral oil used was Atreol No. 9, a light mineral oil, U.S.P., prepared by the Atlantic Refining Co.[7]

[6] Smolens, J., Halbert, S. P., Mudd, S., Doak, B. W., and Gonzalez, L., *J. Immunol.,* in press.

[7] Halbert, S. P., Smolens, J., and Mudd, S., *J. Immunol.,* 1945, **51**, 39.

§ This strain was originally received as a V strain. However, it has been typed by Dr. A. J. Weil using both absorbed serums and "pattern agglutination" technic.[8] He designates the primary antigens as III(IV).

[8] Weil, A. J., Black, J., and Farsetta, K., *J. Immunol.,* 1944, **49**, 321, 341.

‖ The strains of *M. phlei* were kindly supplied by Dr. J. Freund and Dr. H. E. Morton, and the strain of *M. butyricum* by Dr. W. Friedewald.

from the ear veins prior to immunization, and at appropriate intervals thereafter, the serum from each group being pooled. The fold increase in mouse protective titers is represented in Fig. 1.

In a total of 4 such experiments, with varied dosage, or with soluble antigen[6] replacing the killed bacteria, 3 showed essentially the same sort of pattern. That is, the elevation of antibody titer was markedly prolonged by the oil emulsion menstruum and the peak titers were somewhat higher than the saline control groups. In no instance did the alum menstruum bring about maintenance of high antibody levels.

Summary and Conclusions. Investigations have been carried out with a saline-in-mineral-oil emulsion vaccine containing dysentery antigen. The results in mice and rabbits reveal a striking improvement in the antibody response with this menstruum, especially as far as prolongation of high titers is concerned. There was often a definite increase in the peak titers as well. These results have been obtained in the absence of acid-fast bacilli.

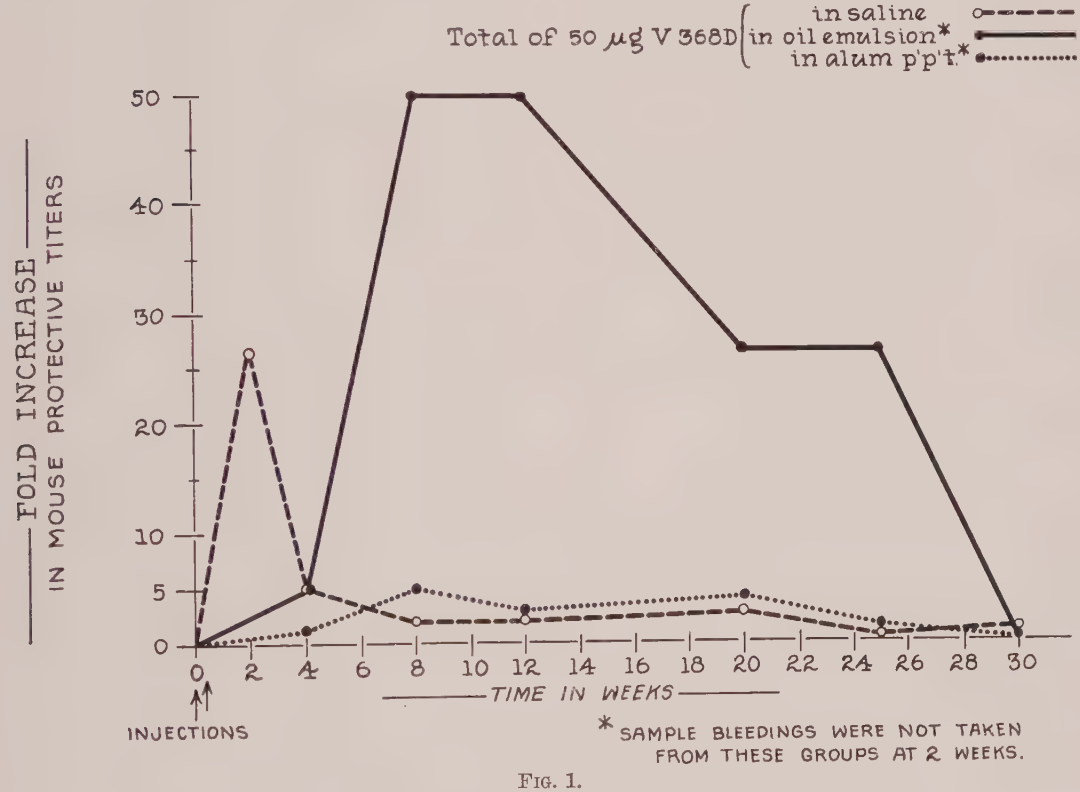

FIG. 1.
Comparative Antigenicity for Rabbits of *S. paradysenteriæ* Killed Bacteria.

15080

Elevation of Plasma Amino Nitrogen as an Index of the Gravity of Hemorrhagic Shock.*

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In a previous report[1] it was shown that a rise in the amino nitrogen content of blood and plasma was a characteristic feature of the later stages of peripheral circulatory failure. This increase in amino nitrogen was attributed to the combined effects of an increased rate of protein breakdown in the peripheral tissues and a decreased ability of the liver to dispose of amino acids. In that report it was shown that the change in amino nitrogen occurred primarily in the plasma, and the data suggested that the amino nitrogen content of the plasma might be useful as a prognostic index in shock. The present report provides additional data on the distribution of amino nitrogen between the erythrocytes, plasma and whole blood during hemorrhage and on the close correlation between the rise in plasma amino nitrogen and mortality following hemorrhage.

Methods. Male rats of the Sprague-Dawley strain, weighing 180-250 g, were used. The animals were fasted 24 hours before the start of bleeding. Amino nitrogen of whole blood, plasma and sedimented red cells was determined by the method of Frame, Russell and Wilhelmi[2] and hematocrits by the method of Meyerstein.[3]

Results. The effect of hemodilution on whole blood amino nitrogen. Amino nitrogen distributions between whole blood, plasma and red cells were determined in a series of 4 rats during hemorrhage, in order to establish which phase most accurately indicated the state of shock. The rats were bled from the tail 2.5 ml per 100 g of body weight in 1 hour and then returned to their cages, where they were allowed water *ad libitum*. One hour later an additional 1 ml was removed, and every 2 hours thereafter 1 ml of blood was taken until the 4 rats died 13.5, 14, 21, and 21.5 hours respectively from the onset of bleeding. By this method a large degree of hemodilution could develop before fatal shock occurred. Fig. 1 illustrates the changes in one rat. The data from the other 3 animals were entirely comparable. It will be noted that until the twelfth hour the whole blood amino nitrogen paralleled the hematocrit while the red cell and plasma amino nitrogen showed little change until after the tenth hour, when rises occurred. The sharp rise in plasma amino nitrogen which took place when severe shock developed after the twelfth hour was then accurately reflected in the whole blood amino nitrogen, since the hematocrit remained low, and the red cell amino nitrogen showed no significant change. These results show that fluctuations in the hematocrit cause changes in the whole blood amino nitrogen content solely because the red cells contain 2.5 to 3 times as much amino nitrogen per unit of volume as does the plasma. For prognostic purposes, therefore, it seemed best to depend on plasma amino nitrogen concentrations.

Rise in plasma amino nitrogen as an index of the gravity of shock. A series of 74 rats kept in a constant temperature room at

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Yale University.

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1 Engel, F. L., Winton, M. G., and Long, C. N. H., *J. Exp. Med.*, 1943, **77**, 397.

2 Frame, E. G., Russell, J. A., and Wilhelmi, A. E., *J. Biol. Chem.*, 1943, **149**, 255.

3 Meyerstein, W., *J. Physiol.*, 1942, **101**, 1.

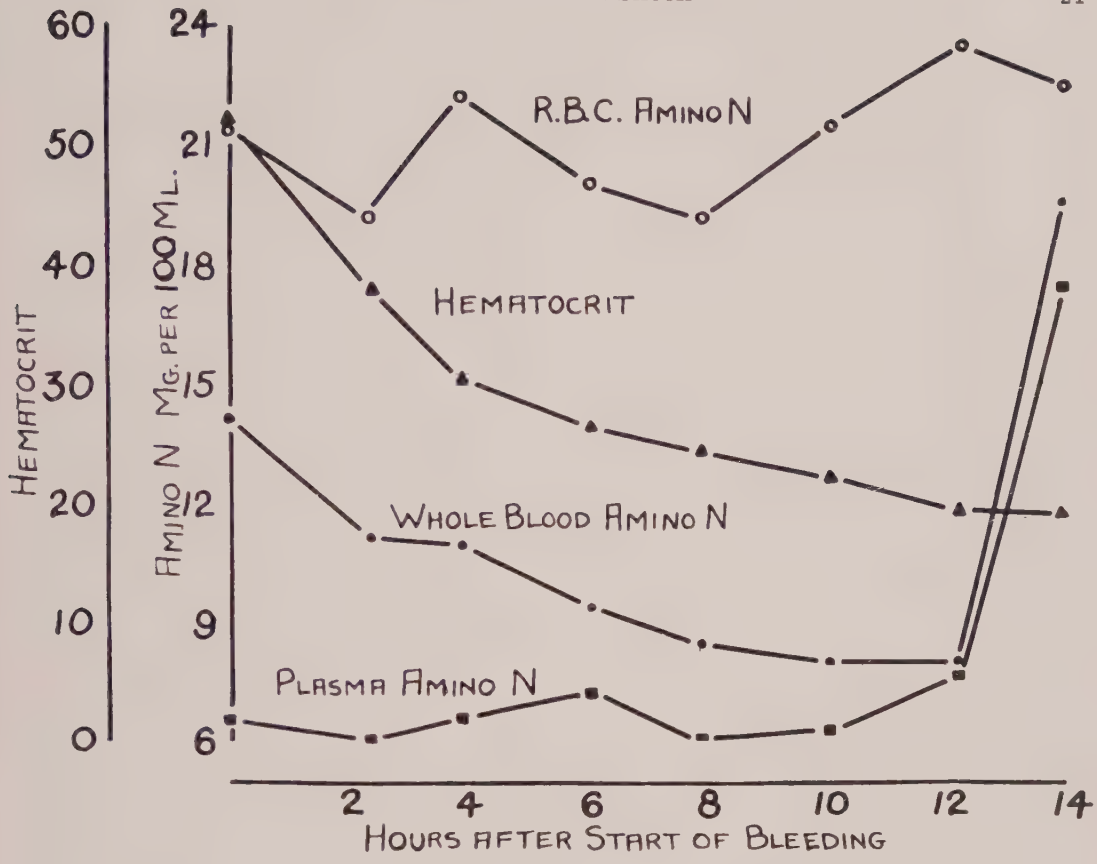

FIG. 1.
The hematocrit and amino nitrogen levels of the whole blood, R.B.C., and plasma of a rat during the course of a prolonged fatal hemorrhage.

83-85° F were suspended in slings according to the technic of Haist and Hamilton[4] and bled from the tails. A volume equal to 2.5 to 2.7 ml per 100 g of body weight was removed over the course of one hour, and plasma amino nitrogen determinations were made at the beginning and the end of the bleeding. No further bleeding was done, and the animals were observed until they died, or for 24 hours if they survived. No water was allowed. A high mortality rate could be expected at the high environmental temperature used. Advantage was taken of the wide variation in survival characteristic of bleeding experiments of this type, in order to correlate the outcome with changes in plasma amino nitrogen. Among those that failed to survive, death occurred from 2 to 140 min after the end of bleeding with an average survival time of 35 min, but there was no correlation between the length of survival and the degree of rise in plasma amino nitrogen during the hour of bleeding. The results in Table I show, however, that there was a good correlation between the proportion of animals surviving and the amount of change in plasma amino nitrogen. When there was a rise of 1.0 mg % or more in amino nitrogen during the hour of bleeding, death invariably occurred if no treatment was instituted. Lesser rises give no sure indication of the outcome but may be useful in indicating a trend toward survival or death if the animals must be sacrificed for experimental purposes.

Discussion. The second group of experiments cited above shows that a rather small rise in plasma amino nitrogen after hemorrhage is a reliable sign that the animal is not

[4] Haist, R. E., and Hamilton, J. I., *J. Physiol.*, 1944, **102**, 471.

TABLE I.
Relation Between the Change in Plasma Amino Nitrogen During Bleeding and the Survival of Rats.

Change in plasma amino nitrogen,* mg %	No. rats	Died	Survived	% died	Survival time in min.†
—1.0 to —0.1	9	3	6	33	55 to 140 (Avg 84)
0 to +0.5	20	11	9	55	5 to 72 (Avg 36)
+0.5 to +1.0	19	14	5	74	6 to 65 (Avg 33)
+1.0 to +5.6	26	26	0	100	2 to 120 (Avg 35)
	74	54	20	73	

All animals bled 2.5-2.7 ml per 100 g of body weight in 1 hour.
* Change in plasma amino nitrogen from beginning to end of hemorrhage.
† Survival time of animals that died only are included. Time in minutes after end of bleeding.

going to survive unless treatment is instituted. The course of the changes in blood amino nitrogen in the single rat cited in Fig. 1 indicates that small but significant increases in plasma amino nitrogen might be missed if the determinations are made only on whole blood. Due to the changes in hematocrit and red cell amino nitrogen in the first 2 hours after start of bleeding, the whole blood amino nitrogen would change by —1.8 to +1.1 mg % for a rise in plasma amino nitrogen of from 0.8 to 5.6 mg %. (It may be noted that the plasma amino nitrogen of the rat represented in Fig. 1 fell slightly by the second hour after start of bleeding, and that this animal survived not only the initial bleeding but several additional hemorrhages. This is consistent with the relation between change in plasma amino nitrogen and likelihood of survival illustrated by the data of Table I.) A rise in plasma amino nitrogen does not mean that the animal cannot be resuscitated if adequate therapy is instituted. Many rats have recovered after receiving transfusions at a time when their amino nitrogen levels were several times normal and the animals appeared moribund.

The change in plasma amino nitrogen levels during hemorrhage and other shock-inducing procedures is recommended as a reliable method of estimating the severity of shock before therapy is instituted or if it is desired to sacrifice the animal for other studies at any given time after the shock-inducing measure has been instituted. Russell *et al.*[5] made use of a similar procedure when studying the oxygen consumption of liver slices at various times after hemorrhage in rats.

Summary. Serial determinations of the hematocrit and the whole blood, plasma and red cell amino nitrogen concentrations were carried out during prolonged hemorrhage and shock in rats. It was shown that, due to the high concentration of amino nitrogen in the red cell, as compared to the plasma, the whole blood amino nitrogen fluctuates with the hematocrit, while the plasma amino nitrogen more closely reflects the degree of shock. In a large series of rats a rise in plasma amino nitrogen of 1 mg % or more after a 2.5 to 2.7 % hemorrhage was invariably associated with a fatal outcome.

[5] Russell, J. A., Long, C. N. H., and Wilhelmi, A. E., *J. Exp. Med.*, 1943, **79**, 23.

15081

Effect of Penicillin on Bacterial Contamination of Eggs and Tissue Cultures Inoculated with Unfiltered Sputums.

HARRY M. ROSE, ELEANORA MOLLOY, AND ELIZABETH O'NEILL. (Introduced by A. R. Dochez.)

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The isolation of viral agents from the human respiratory tract, by the direct inoculation of tissue cultures or chick embryos with sputum or nasopharyngeal washings, is hindered by various bacteria which are invariably present in the nose and throat, and it is common practice to remove these contaminating microorganisms by filtering the sputum or washings before inoculation. At the same time, however, so much virus may also be lost that the filtrates are non-infective, as Hirst[1] showed very well in the case of influenza virus, and for this reason attention has recently been directed to the use of technics in which filtration is avoided. Rickard, Thigpen and Crowley[2] found that chick embryos inoculated with untreated and unfiltered throat washings from cases of influenza usually survived 48 hours, and that the virus, when present, developed normally in spite of heavy contamination of the egg fluids with a variety of bacteria from the nasopharynx. Eaton, Corey, Van Herick, and Meiklejohn[3] also isolated influenza virus directly in eggs from unfiltered throat washings, and attempted to prevent bacterial contamination of the embryos by preliminary treatment of the washings with Zephiran;[4] but they found that this agent, in concentrations which would not affect the virus, was not consistently effective in removing the microorganisms. More recently, Hirst[5] reported that the inoculation of unfiltered throat washings together with penicillin into the amniotic sac was the most sensitive method of isolating influenza virus in eggs, and that penicillin would satisfactorily control bacterial contamination without interfering with the virus.

In line with these studies we have studied the effect of various antibiotic agents on the bacterial contamination of tissue cultures and chick embryos inoculated with unfiltered sputums, with the purpose of developing a method whereby filtration, with consequent loss of viral agents, could be avoided, but bacterial contamination either prevented or held to a minimum. Preliminary experiments indicated that sulfadiazine, even in high concentrations, was inadequate as a bacteriostatic agent. Zephiran interfered with the development of influenza virus in amounts that controlled bacterial growth. Propamidine and pentamidine were found to be lethal for chick embryos and for tissues in culture. Penicillin, however, gave more favorable results, which form the substance of this report.

Experimental. Fifty-five specimens of sputum from patients with infections of the respiratory tract were examined. Most of these patients had pneumonias of bacterial etiology, or primary atypical pneumonia. A few had an acute catarrhal bronchitis. Two persons exhibited a severe gingivo-stomatitis suspected of being herpetic in origin.

All of the sputums were freshly collected and immediately suspended in equal volumes of physiological saline or nutrient broth by grinding with an abrasive in a mortar, followed by centrifugation at 3000 rpm for 20 minutes in an angle-head centrifuge. The supernatant fluids were then divided into 2 parts, one of which was not treated, while penicillin was added to the other to give a

1 Hirst, G. K., *J. Immunol.*, 1942, **45**, 293.

2 Rickard, E. R., Thigpen, M., and Crowley, J. H., *J. Immunol.*, 1944, **49**, 263.

3 Eaton, M. D., Corey, M., Van Herick, W., and Meiklejohn, G., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 6.

4 Personnel of U. S. Naval Laboratory Research Unit No. 1, *Science*, 1942, **96**, 53.

5 Hirst, G. K., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 155.

TABLE I.
Effect of Penicillin on Bacterial Contamination of Tissue Cultures Inoculated with 55 Unfiltered Sputums.

A	
Single tissue cultures inoculated with untreated sputum.	
Sterile	Contaminated
2	53

B		
Paired tissue cultures inoculated with sputum plus penicillin.		
Both sterile	One sterile One contaminated	Both contaminated
31	1	23

TABLE II.
Effect of Penicillin on Bacterial Contamination of Chick Embryos Inoculated with 55 Unfiltered Sputums.
A. Chick Embryos Inoculated with Untreated Sputum (one egg each specimen).

Days of incubation	Survived		Died	
	Sterile	Contaminated	Sterile	Contaminated
1	—	—	None	47
2	—	—	—	1
3	—	—	—	—
4	—	—	—	1
5	6	None	—	—

B. Chick Embryos Inoculated with Sputum Plus Penicillin (2 eggs each specimen).

Days of Incubation	Both embryos survived (34 specimens)		One embryo survived, one died (13 spec.)				Both embryos died (8 spec.)	
			Survived		Died			
	Sterile	Contaminated	Sterile	Contaminated	Sterile	Contaminated	Sterile	Contaminated
1	—	—	—	—	4	4	3	2
2	—	—	—	—	2	1	3	—
3	—	—	—	—	—	—	—	—
4	—	—	—	—	1	—	2	2
5	68	None	13	None	—	1	3	1

concentration of 500 or 1000 Oxford units per milliliter. The dilution of the treated supernates, as the result of adding the solutions of penicillin, was negligible.

Three tissue cultures, prepared from chopped chick embryo and a modified Simms salt-serum solution,[6] were inoculated with each sputum, one with 0.5 ml of the untreated suspension, and 2 with 0.5 ml of the suspension containing penicillin. The cultures were incubated at 37°C and inspected daily; when turbidity and changes in pH occurred, gram-stained smears were examined microscopically and subcultures were made on rabbits' blood agar.

Each sputum was also inoculated to the amniotic sac of three 11-day-old chick embryos, one receiving 0.2 ml of the untreated suspension, and two 0.2 ml of the suspension containing penicillin. The eggs were incubated at 37° C and candled every 24 hours. When embryos appeared to have died the eggs were opened at once and smears and subcultures made of the allantoic fluids. Embryos that survived were held for 5 days after inoculation and then examined in the same manner.

The results with the tissue cultures are given in Table I. Fifty-three (96.3%) of the 55 single tissue cultures inoculated with untreated sputums showed gross bacterial contamination within 24 hours. Only 23 (41.8%) of the paired cultures inoculated with the same sputums plus penicillin became contaminated, while 31 (56.3%) remained

[6] Rose, H. M., Culbertson, J. T., and Molloy, E., *J. Parasitol.* (Suppl.), 1944, **30**, 16.

sterile for 5 days. With one sputum treated with penicillin, one culture was contaminated and the other sterile.

Differences between the results obtained with treated and untreated sputums were more marked in the eggs than in the tissue cultures, as illustrated in Table II. Forty-nine (89.1%) of the 55 single chick embryos inoculated with untreated sputums died as the result of bacterial contamination, 47 within 24 hours, while either one or both embryos of 47 pairs (85.5%) inoculated with sputum and penicillin survived for 5 days. All of the surviving eggs were found to be bacteriologically sterile.

A variety of microorganisms were recovered from the eggs and tissue cultures that showed bacterial contamination despite the addition of penicillin. In the main these proved to be gram negative bacilli of the Proteus or Pseudomonas groups, together with a number of coliform organisms that were not further identified. Several penicillin resistant strains of staphylococci were also encountered.

Discussion. We observed that the inoculation of fresh unfiltered sputums to developing hens' eggs was almost invariably followed by gross bacterial contamination and death of the embryos within 48 hours. The addition of penicillin to the sputums, however, prevented bacterial growth and permitted survival of the great majority of the embryos. The implication of these findings in respect to the isolation of viral agents from the sputum by direct egg inoculation is apparent.

Herpes virus was isolated on first passage in eggs inoculated with penicillin-treated sputum from the 2 cases of gingivo-stomatitis referred to earlier in this paper. This is the first successful isolation of herpes virus directly in chick embryos and will be described in detail in a separate communication. It is of interest that eggs inoculated with the untreated sputums of the same cases died within 24 hours with failure to recover the virus.

Unfortunately, because of the recent dearth of clinical material, we have been able to examine throat washings from only one case of serologically proved influenza by the method described. In this single instance, however, influenza A virus was readily isolated in eggs by allantoic inoculation of the washings treated with penicillin. Subsequent study of this virus strain in egg passage was greatly facilitated by the absence of bacteria contaminating the allantoic fluids.

In contrast to the favorable results obtained in chick embryos, penicillin failed to prevent bacterial contamination in a large proportion of cultures inoculated with unfiltered sputums.

Summary. The addition of penicillin to unfiltered sputums inoculated to chick embryos prevented bacterial contamination and permitted survival of the embryos in the majority of instances. Less favorable results were obtained in tissue cultures.

Reference is made to the isolation of 2 strains of herpes virus by primary egg inoculation of unfiltered sputums treated with penicillin.

15082

Effects of Temperature on the Excised Frog Heart.*

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In a previous manuscript[1] it was reported that when the excised frog heart was placed in a controllable modulated high frequency field there resulted either reversible cardiac standstill or cardiac standstill followed by a type of cyclic activity. The cause or causes for such modified activity we were only able to conjecture.

The purpose of the present study was to see if similar results could not be obtained by the application of physical heat and to ascertain the influence of atropine and eserine upon the heat responses of the excised preparations since atropinized and eserinized preparations respond differently to the condenser field.[2]

The heart was arranged for perfusion as shown in Plate 1. The Ringer's solution from the perfusion bulb first passed through the heating bath d and then to the constricted portion of the heart cannula h where it could enter the ventricular chamber during diastole and in turn be forced back through the heart cannula during systole and eventually through the over-flow pipes spraying the outside of the heart. The temperatures recorded were those shown by the thermometer g, the bulb of which was immersed in the perfusion fluid in chamber e. This method allowed for constant perfusion pressure and for constant volume flow over the outside of the heart. Employing this method for perfusing the heart one of 3 variables was then introduced and kymographic records of heart activity recorded in each instance: The 1st variable introduced consisted of changing the temperature of the perfusion fluid going to the heart (controls); the 2nd consisted of changing the temperature of the perfusion fluid after atropine 1-50,000 had been added; the 3rd consisted of changing the temperature of the perfusion fluid after eserine 1-125,000 had been added.

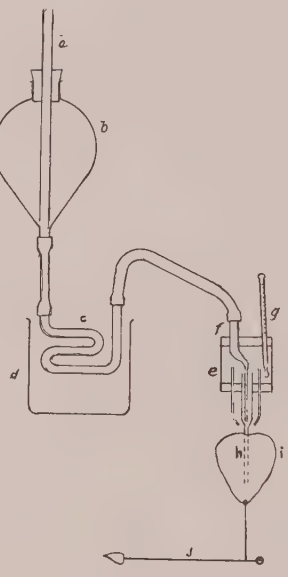

PLATE 1

Schematic illustration of perfusion apparatus. For description see text.

Results. Studies were made of records from 63 hearts, equally divided among the controls, atropinized and eserinized preparations. It was found that as the temperature of the perfusion fluid was slowly increased there developed a period of irregular activity which commenced at the average temperature of 29° C for the controls and for the atropinized hearts, and at 28° C for the eserinized preparations. The irregularity which developed at the above average temperatures and which persisted until a higher temperature had been attained in each instance, is difficult to describe because of the many dif-

* Aided in part by a grant from the University of Utah Research Fund.

1 Fenning, Con, and Mott, Clarence R., *Am. J. Physiol.*, to be published.

2 Fenning, Con, and Mott, Clarence R., in preparation.

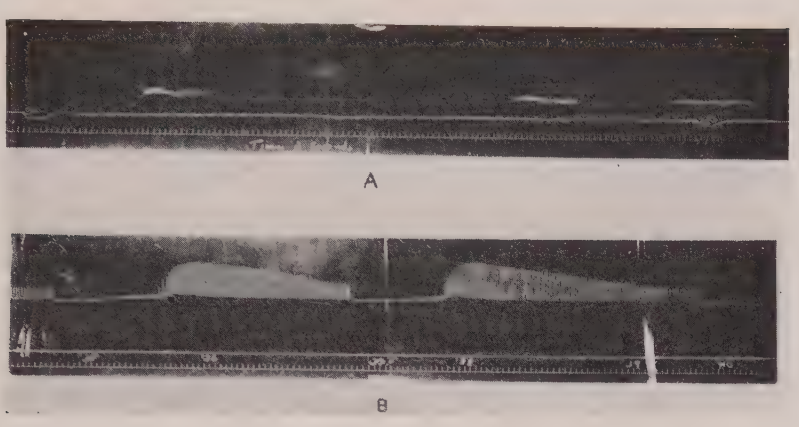

FIG. 1.
Shows types of kymographic records obtained from the excised frog heart when the temperature of the perfusion fluid was the only factor varied. (Controls.)

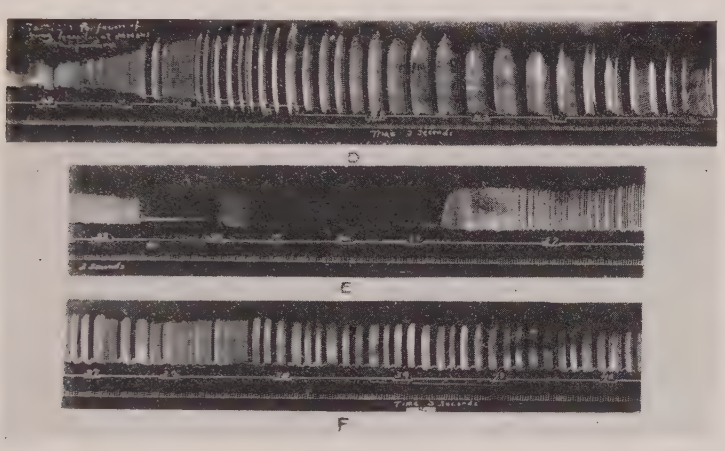

FIG. 2.
Shows types of kymographic records obtained from the excised frog heart when the temperature of the perfusion fluid was the only factor varied. (Controls.)

ferent patterns in which it presented itself. We have observed brief ventricular stoppage with the atria continuing their activity (Fig. 2 E); an abrupt 100% increase in rate; changes in amplitude and rate (Fig. 2 D); complete but momentary cardiac standstill and many other patterns. However in all instances there developed subsequently (average temperature of 32.6° C for the controls, 38.5° C for the atropinized and 37° C for the eserinized preparations) a type of cyclic activity which varied widely from preparation to preparation and, furthermore, may have varied from moment to moment in the same preparation as shown in Fig. 1 and 2 for the controls, Fig. 3 for the atropinized hearts and Fig. 4 for the eserinized preparations. M and N of Fig. 4 are continuations of L. A 7-minute period of the record in which no activity occurred was removed between L and M and again between M and N. In all the eserinized preparations marked tonus, or tonus-like, changes occurred between the periods of activity as noted on the records of Fig. 4. These characteristic tonus changes were not observed in the atropinized or control hearts.

The cycles which came on at the above stated temperatures would persist for some time providing the temperature was maintained at the proper critical level. We have observed continuance of cyclic activity under

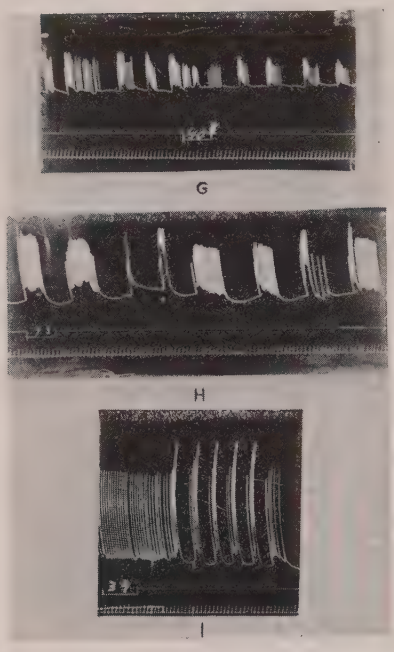

FIG. 3.

Shows types of kymographic records obtained from the excised atropinized frog heart when the temperature of the perfusion fluid was varied.

the above conditions for a period of two hours and there is no reason to believe it would not have continued longer. In all cases rhythmic activity was restored when the temperature was lowered. Occasionally it was observed that once cycles had been well established the temperature could be lowered 2-4 degrees below the temperature at which they commenced and still maintain continuance of cyclic activity. In other preparations apparently adaptation occurred and in order to maintain cycles it was necessary to raise the temperature a few degrees above that at which they first commenced. It should be pointed out that in the control hearts especially there was a tendency for the ventricle to become cyclic (Fig. 1B; 2E,F) while the atria remained active. When the temperature was raised somewhat higher, however, the atria too would become cyclic. This phenomenon was observed occasionally in the atropinized hearts but not in the eserinized preparations.

When the perfusion fluid temperature was increased to about 45° C activity would cease except for a slowly developing heat response which was also reversible providing the temperature was subsequently lowered. In no instance with the above approach did we obtain cycles which resembled in all respects those induced by the modulated high frequency condenser field, although cycles occurred in 100% of the hearts studied. Just why cycles occurred in these preparations and why the onset temperature differed with atropine and with eserine we are at present unable to state.

A second and perhaps more drastic approach was then instigated as follows: The heart was arranged for perfusion as above shown. The perfusion fluid passing to the heart was either stopped or maintained at room temperature, and then from 5-10 cc of Ringer's solution brought to temperatures varying between 40-95° C was poured over the outside of the heart. Cyclic activity of short duration was induced by this approach in most instances and could be made to appear at any temperature between 40-95° C. Fig. 5-O shows the usual response: Immediate cessation of activity, followed by an abrupt rise in tone (heat response) which slowly declined and usually one or more periods of cyclic activity supervened before the return to apparently normal rhythmic activity. What appeared to be sinus tachycardia frequently occurred at the very peak of the heat response. Again there may have been cardiac arrest followed by the heat response but with no cyclic activity occurring between the time the hot Ringer's was applied and the return to apparently normal activity. In one instance following the application of 95° C Ringer's to the heart the usual heat response occurred and there then supervened a period of 90 minutes before activity commenced.

Attention is called to the record shown in Fig. 5 P which was obtained after a continuous recording of 96 minutes during which time the heart was subjected to Ringer's heated to 80° C 3 different times, to Ringer's brought to 55° C once, and to Ringer's at 40° C once, following which there occurred the type of activity shown. There were 7

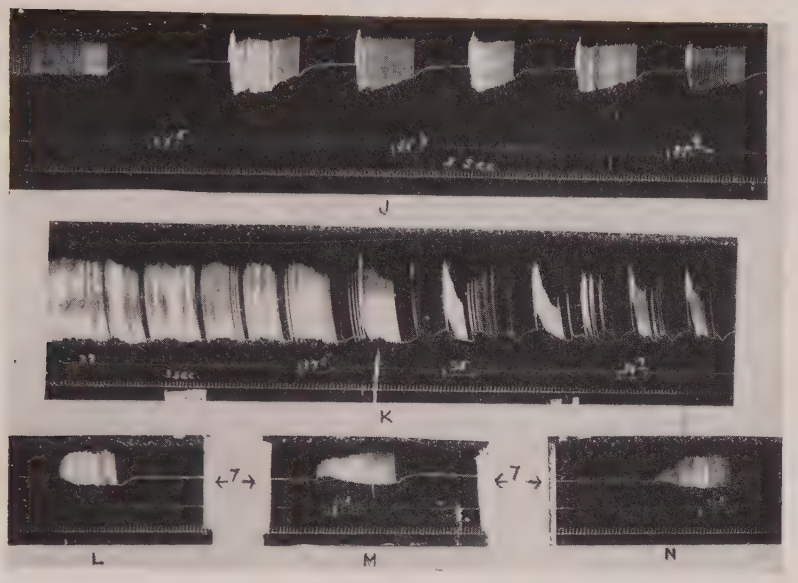

FIG. 4.
Shows types of kymographic records obtained from the excised eserinized frog heart when the temperature of the perfusion fluid was varied.

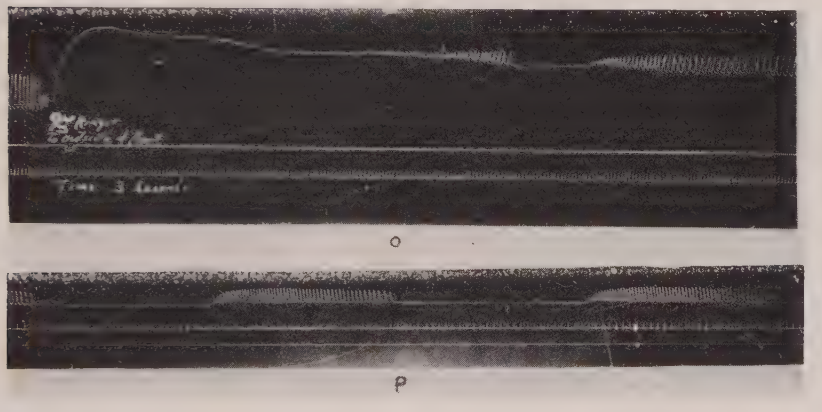

FIG. 5.
O shows the type of response obtained when hot Ringer's is applied directly to the outside of the heart. P shows a type of cycle which resembled those obtained when the frog heart was placed in modulated high frequency field.

such periods of intermittency before the return of continuous activity. The cycles were regular in manner of onset, manner of stoppage, duration of activity, duration of standstill and in all respects resembled one type of cycle brought about by the modulated high frequency condenser field mentioned above except they did not persist as long.

Conclusions. Atropine and eserine apparently raise the critical temperature threshold for the thermal responses of the excised frog heart preparation. Differentially, atropine has a greater effect than eserine. The response of the excised frog heart to a slowly rising temperature does not warrant the assumption that the modulated high frequency condenser field operates entirely in the same manner.

15083

Observations on the Antigenicity of Viridans Streptococci Isolated from the Air.*

MATHILDE SOLOWEY. (Introduced by B. C. Seegal.)

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In a previous report[1] it was noted that viridans streptococci were found widely distributed in the air of enclosed occupied places as well as in the open air. Many of these organisms seemed to be identical in morphology and biochemical characteristics with those recovered from the human nasopharynx and were therefore designated "typical." A group of "atypical" streptococci also were recovered from the air, which differed in some aspects of their morphology and biochemical characteristics from the viridans streptococci usually isolated from human throats. The principal differences noted were that the atypical organisms usually appeared as diplococci, tetrads, irregular groups, or very short chains; also these streptococci formed more opaque colonies, tended to grow with more uniform turbidity in broth culture, and when grown in 0.5% dextrose broth produced less acid.

We have described a serological classification of the viridans streptococci of human origin.[2] The present study is concerned with the serological grouping of viridans streptococci isolated from the air in order to compare the distribution of serologically identifiable groups of air streptococci with that already determined for organisms isolated directly from human sources.

Methods and Procedures. Exposure Locations: Samples from street air were obtained by exposing sheep's blood agar plates for one hour on the steps outside of a building on a busy street.

Samples from an occupied classroom of the amphitheatre type were obtained by exposing plates on several levels throughout the room. Although the plates were exposed for periods ranging from one to 3 hours before, during and after occupancy, the total bacterial counts were low. By exposing the plates in the pit of the amphitheatre and setting in motion 2 rotating electric fans 2 or 3 feet to one side and above the plates, a satisfactory seeding of the plates was obtained. The total bacterial counts per plate increased as did the number of viridans streptococci.

Treatment of Cultures. All the plates were incubated 48 hours at 37° C and total counts as well as counts of suspected viridans streptococci were made. Single colonies of these latter were picked off into nutrient broth containing one drop of sheep's blood. After incubation for 18-24 hours at 37° C, they were examined for purity and tested biologically for classification as "typical" or "atypical." The strains then were tested by means of the precipitin reaction with antiserums for 4 serological groups, I, II, III, and IV, previously prepared by immunizing rabbits with strains of viridans streptococci isolated from bacterial endocarditis. The technic for this as well as for the preparation of the antigen extract and the precipitin test have been reported previously.[2]

Results. Fifty strains of "typical" viridans streptococci were obtained, 47 of which were found on the plates exposed in the occupied classroom. Twenty-eight or 56% gave positive reactions with antiserums of either groups I, II, III, or IV; 10 reacting with group I, 7 with group II, 5 with group III, and 6 with group IV antiserums. In earlier studies,[2,3] it was reported that a little more than 50% of the strains from bacterial endocarditis, human throats and extracted teeth, and acute respiratory infections were classi-

* Supported by a grant from the J. B. Pierce Foundation.

1 Buchbinder, L., Solowey, M., and Solotorovsky, M., *Am. J. Pub. Health*, 1938, **28**, 61.

2 Solowey, M., *J. Exp. Med.*, 1942, **76**, 109.

3 Solowey, M., *Am. J. Hyg.*, 1944, **39**, 295.

fiable into the same 4 groups.

Thirty-nine "atypical" strains of viridans streptococci were examined, 30 of which came from plates exposed to the street air, and 9 from plates exposed to the classroom. Six or 15% gave reactions with one of the 4 groups, 3 falling into group I, none into group II, I into group III, and 2 into group IV. One of the 6 reacting strains was recovered from the classroom air, the remaining 5 from the street air.

Discussion. More than half of the "typical" viridans streptococci isolated from the air were classifiable antigenically by the use of antiserums obtained by immunizing rabbits with strains of streptococci from bacterial endocarditis. Immunologically therefore they resemble the viridans streptococci obtained from human sources, 50% of which are classifiable antigenically with the same group antiserums. The "atypical" streptococci obtained from the air less frequently show antigenic relationship with the human strains.

It has been suggested that the presence of viridans streptococci in the air may be used as an index of human contamination in the same manner as the presence of *E. coli* is used as an index of fecal contamination. A method has been offered by which it is possible to identify many viridans streptococci serologically. The procedure may possibly be applied in epidemiological studies in the identification of viridans streptococci of human origin.

Summary. Twenty-six of 50 "typical" streptococci isolated from the air resemble immunologically the viridans streptococci obtained from human sources, whereas only 6 of 39 "atypical" streptococci from the air exhibit a similar relationship.

The epidemiological application of the data is discussed.

15084

Complement Fixation Antigen in *Entamoeba histolytica* Infection.

JOHS. BÖE. (Introduced by Konrad Birkhaug.)

From Kaptein W. Wilhelmsen og Frues Bakteriologiske Institutt, Oslo, Norway.

Although infection with *Entamoeba histolytica* produces specific antibodies, the serological diagnosis is scarcely employed in this disease because of the ease with which ameba are demonstrated microscopically, thus rendering a serological diagnosis superfluous. However, the main obstacle to an extended use of a serological reaction has been the extreme difficulty of preparing a useful antigen.[1]

Izar[2] demonstrated specific antibodies in *E. histolytica* infection and obtained positive complement-fixation reactions during the disease as well as in those whom he considered to be healthy carriers. As antigen he employed an aqueous extract of ameba-containing feces and the contents of a liver abscess. Craig[3] however first put the serological diagnosis in amebic infections upon a rational basis. He made use of an alcoholic extract of ameba-cultures as antigen. But this extract was quite weak and had to be used undiluted. Craig found that the serological reaction was quite often positive in *E. histolytica* infection and exhibited a high degree of specificity. But the serological diagnosis often failed in cases where it would be most useful, namely in ulcerative colitis. The findings by Craig have since been confirmed by many, including Tsuchyia[4] and Weiss and

1 Manson-Bahr, P., *The Dysenteric Disorders*, Cassell & Co., London, 1939.

2 Izar, G., *Arch. Schiffs. u. Tropenhyg.*, 1914, **18**, Beiheft II.

3 Craig, C. F., *Amebiasis and Amebic Dysentery*, Chas. F. Thomas, Balto., 1934.

Arnold[5] who attempted to improve the antigen preparation. Otherwise, the serological reaction was conducted as the Wassermann reaction or with slight modifications.

Paulson and Andrewes[6] made a comparative study of various amebic antigens by sending sera to 3 different laboratories, after a thorough parasitological examination of the patients. The serological findings in these 3 laboratories showed wide variations. Several sera gave positive reactions in cases with negative fecal findings and negative serological reactions in cases with positive amebic findings. But in the main there was agreement between the serological and parasitological findings.

Besides being a diagnostic aid, the serological test may be of some value during the treatment of amebic dysentery. Thus Craig demonstrated that the serological reaction turned negative following successful treatment and turned positive when relapse occurred. But the serological reaction has also certain theoretical interest in the biology of *E. histolytica*. Thus if the "healthy carrier" shows antibody production, then we may suppose, according to our knowledge about other infections, that the ameba has invaded the tissue. Thus Dobell[7] may be correct in considering the *E. histolytica* as an obligate tissue parasite, as opposed to the view of Reichenow[8] and Westphal[9] that the ameba occurs normally only in the intestinal lumen.

In a study of the distribution of pathogenic protozoa in a group of Norwegians[10] we made a special search for intestinal *E. histolytica* and found that approximately 3% of 1100 healthy persons had this parasite in their feces. These ameba proved virulent in cats which presented the characteristic histopathological picture with ameba in the intestinal wall. Our study included also a number of sera for detection of specific amebic antibodies. At first we found it expedient to simplify the antigen preparation, which hitherto appeared to be the main stumbling block in the serological diagnosis of amebic disease. Inasmuch as our Norwegian material is very scanty, we trust that others having greater access to clinical material will repeat our work. Hence we shall detail the preparation of our antigen.

Antigen. E. histolytica was grown on a modified Cleveland-Collier medium. Ten heavy cultures were transferred to a centrifuge tube and centrifuged at low speed. The supernatant fluid was decanted, the residue suspended in saline and re-centrifuged. This process was repeated usually about 8-10 times until the supernatant fluid became clear. The bacteria-poor residue now consisted mostly of ameba plus traces of starch from the medium. The residue was dried thoroughly in a desiccator, pulverized finely in a porcelain mortar and kept dry in ampules. This powder was resuspended in saline and titrated for its anticomplementary dose, one-fourth of which was employed in the actual tests.

Other reagents: The hemolytic system contained a 2.5% suspension of sheep red blood cells and an anti-sheep-erythrocyte-amboceptor with a titre of 1:4000, produced in the rabbit. Fresh guinea pig serum served as complement. The sera to be tested were inactivated at 56° C for one-half hour.

Complement-fixation reaction: The serological technic was identical with the usual Wassermann test. We employed 0.2 cc of each reagent and the total amount of liquid measured 1 cc. Four units of amboceptor and 2 units of complement were used, with the necessary controls. As positive control we employed serum from the rabbit immunized with the saline suspension of the *E. histolytica* antigen. This rabbit serum had a very high titre and gave negative reactions with the contaminating fecal bacteria. After having mixed serum, antigen and complement, the tubes were placed on the water-bath at 37° C for one-half hour. At this

4 Tsuchyia, H., *J. Lab. and Clin. Med.*, 1934, **19**, 495.

5 Weiss, W., and Arnold, *Am. J. Digest. Dis. and Nutr.*, 1934, **1**, 231.

6 Paulson, M., and Andrewes, J., *Arch. Int. Med.*, 1938, **61**, 562.

7 Dobell, C., *Med. Res. Comm. Spec. Rep. Ser.*, 1918, No. 15.

8 Reichenow, E., *Arch. Schiffs. u. Tropenhyg.*, 1937, **41**, 257.

9 Westphal, A., *Ibid.*, 1938, **42**, 441.

10 Böe, Johs., *Acta Med. Scand.*, 1943, **113**, 321.

time we added amboceptor and erythrocytes. After 10 minutes on the waterbath we read the reaction and controlled this after 20 minutes at room temperature. The complement-fixation reaction was also done at ice-box temperature. Our impression was that the reaction proceeded more rapidly and in higher titre. Otherwise our results were essentially the same. We compared these results with those obtained with an antigen prepared exactly after Craig's method. The 2 antigens gave in our hands identical results. A total of 121 sera were tested with the 2 antigens. In only 5 of these patients could we demonstrate *E. histolytica* in the feces. The serological reaction was positive in only one of the healthy amebic carriers as well as in one patient who showed no ameba in the feces. This latter patient had an uncertain liver disturbance, but our numerous fecal examinations remained negative for ameba. Our sole purpose for presenting these data is to induce investigators having access to greater clinical material, to repeat the serological reaction in amebic dysentery with the saline suspension antigen of *E. histolytica.*

Summary. A saline suspension of *E. histolytica* was employed as antigen in the complement-fixation test which gave identical results with those obtained with the Craig alcoholic extract antigen of *E. histolytica.*

15085

Protection of Animals Against *Cl. welchii* (Type A) Toxin by Injection of Certain Purified Lipids.*†

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(Introduced by Joseph C. Aub.)

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This communication reports evidence that purified total lipid extracts of certain animal organs protect mice and dogs against the lethal action of the toxin of *Cl. welchii* (type A).‡ In the case of hog liver, this protective action has been traced to the acetone insoluble, alcohol soluble (lecithin) fraction. Wüth[1] reported that hemolysis of red cells *in vitro* by *Cl. welchii* could be inhibited by lecithin. Since bacterial toxins have been inhibited *in vitro* in a non-specific way by a number of substances, including soaps,[2] olive oil,[3] lanolin,[4] and cholesterol,[5] this observation received little attention, other than mention by van Heyningen[6] that the effect might

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‡ The authors are indebted to Professor Milan A. Logan for the purified *Cl. welchii* (type A) toxin used. This is a glycerol dialyzed toxin, containing chiefly alpha toxin, a small amount of theta toxin (accounting for less than 5 out of a thousand MLD's), and approximately 2000 mucin clot prevention units of hyaluronidase activity per cc.

1 Wüth, O., *Biochem. Z.*, 1923, **142**, 19.

2 Larson, W. P., Halvorson, H. O., Evans, R. D., and Greene, R. G., *Colloid Symposium Monographs*, 1925, Vol. 3, p. 152, New York Chemical Catalogue Co. (Reinhold Publishing Corp.).

3 Myers, G. N., *J. Hygiene*, 1934, **34**, 250.

4 Weinberg, M., and Guillaumie, M., *C. R. Soc. Biol.*, 1935, **120**, 936.

5 Degkwitz, R., *Erg. d. physiol.*, 1931, Bd. 32, S. 821.

6 van Heyningen, W. E., *Biochem. J.*, 1941, **35**, 1246.

be due to substrate competition. MacFarlane and Knight[7] showed that *Cl. welchii* toxin contained an enzyme which hydrolyzed lecithin into phosphorylcholine and a diglyceride. They considered this lecithinase to be probably identical with the alpha or principal toxin of *Cl. welchii* (type A) culture filtrates. Since MacFarlane and Knight did not determine whether the enzymatic action was specifically restricted to lecithin, the possibility must be considered that phosphatide substrates other than lecithin may be hydrolyzed by the alpha toxin. On this particular point we have found that phosphatidyl serine, sphingomyelin, and soy bean phosphatides[8] are not hydrolyzed by the *Cl. welchii* alpha toxin.

Clostridium welchii alpha toxin hemolyzes erythrocytes and destroys tissue cells,[9,10] an effect presumably related to its action on phosphatides of the cell surface. It occurred to the writers that intravenous injection of phosphatides might divide the action of *Cl. welchii* toxin between hydrolysis of the added phosphatides and of cell phosphatides. Provided a sufficiently high concentration of added phosphatides were present, the hydrolysis of the cell phosphatides might be reduced to a minimal figure, by a *substrate partition effect*. The increasing concentration of split products of the reaction might also be expected eventually to decrease the rate of cytolysis.

With this idea in mind, a number of lipid preparations have been tested (a) for inhibitory action of hemolysis by *Cl. welchii* (type A) toxin *in vitro;* (b) for toxicity for mice and dogs on intraperitoneal or intravenous injection; (c) for protective action, on intraperitoneal injection into mice, against *Cl. welchii* toxin injected intraperitoneally by separate injection; and (d) for protective action for mice and dogs when injected intravenously, against *Cl. welchii* toxin administered intravenously from one to 10 minutes after injection of lipids. A number of incidental observations have also been made. The methods used and detailed experimental results are reported below.

Methods. Preparation of purified total lipid extracts: Purified total lipid extracts[11] of erythrocytes have been prepared in 3 slightly different ways: (1) the cells were packed by centrifugation, hemolyzed with an equal volume of distilled water, and the lipids extracted at room temperature by addition of 25 volumes of 1:1 alcohol-ether. After filtration, the extract was concentrated to a small volume in a water suction pump vacuum. The resulting aqueous emulsion was freed of nonlipid impurities by dialysis through Visking cellophane membranes against distilled water at approximately 3° C. After dialysis, the emulsions were lyophilized. (2) The procedure was the same as (1) with the exception that 8 volumes of 1:1 alcohol-ether were used instead of 25 volumes. (3) 100 cc portions of packed erythrocytes were homogenized for 45 seconds in a Waring blendor, 200 cc of alcohol were added, the mixture homogenized for one minute, 200 cc of ether were added, and the homogenization allowed to proceed for another minute. The mixture was filtered through a Buchner funnel and the clear alcohol-ether filtrate handled as described above. The 3 methods yield apparently identical purified total lipid extracts of erythrocytes.[8]

Purified total lipid extracts have been prepared from various other sources by the method described under (3), unless otherwise stated. The best source of protective lipids found so far is liver, which yields approximately 30 g of purified total lipids per kilo of wet tissue, whereas erythrocytes yield only 3-4 g per kilo of packed cells.

The lipid preparations were injected as emulsions (either in 0.9% sodium chloride or distilled water), ranging in concentration from 2 to 10%. The emulsions were made by addition of distilled water to the dried lipid preparations, followed by stirring or rotation until a homogenous emulsion was present. In the case of hog liver lipids so pre-

[7] MacFarlane, M. G., and Knight, B. C. J. G., *Biochem. J.*, 1941, **35**, 884.

[8] Unpublished data.

[9] Bull, C. G., and Pritchett, I. W., *J. Exp. Med.*, 1917, **26**, 119.

[10] Glenny, A. T., Barr, M., Llewellyn-Jones, M., Dalling, T., and Ross, H. E., *J. Path. and Bact.*, 1933, **37**, 53.

pared, the state of the emulsion was improved by adjustment of the pH from 6.2 to 7.5 by careful addition of normal sodium hydroxide. Prior to injection into animals, emulsions were always centrifuged to remove minute amounts of material not well emulsified.

Fractionation of lipid extracts. To a 10% solution of total lipids in chloroform 6 volumes of acetone were added. The mixture was centrifuged and the supernatant collected (acetone soluble fraction). The precipitate was dissolved in half the volume of chloroform used previously, and the solution was treated with a 6-fold excess of alcohol. The mixture was centrifuged and the precipitate (alcohol insoluble or cephalin fraction) collected separately. Aliquots of the 3 fractions thus obtained were combined in such proportions as to reproduce the mother material. The fractions and the recombined mixture were freed of solvents by means of a vacuum water pump, and emulsified as described above.

Technics used in testing for protective effect. As a preliminary screen test, in an effort to sort out ineffective preparations from promising ones, an *in vitro* hemolysis test has been used, as follows: 0.1 cc fresh, washed canine erythrocytes, 1.0 cc 0.1M borate buffer (Sorensen) of pH 7.5, 0.02 cc toxin (containing 36 LD_{50}), 10 cc 0.003 M $CaCl_2$ in 0.9% sodium chloride, 0.2 cc 5% lipid. An Evelyn photoelectric colorimeter, with a 660 $\mu\mu$ filter, was used to measure changes in optical density[12] occurring during the hemolytic reaction. The reaction was carried out at room temperature (24° C) over a one hour period, in a series of colorimeter tubes with included appropriate controls. Colorimeter readings were taken at 10-minute intervals, the tubes being well mixed by inversion just prior to each reading.

The colorimetric test was checked against direct observation of the rate of hemolysis using a microscope counting chamber, and good agreement was obtained. The latter has been found to be a sensitive but tedious method of following the hemolytic reaction.

Animal testing. Strain A mice or market mice weighing 20 to 30 g were injected intravenously by tail vein, or intraperitoneally, with the more promising preparations. The LD_{100} of toxin was determined to be 4.2 LD_{50} when given intravenously as follows: in 3 divided doses of 0.14 cc each of a 10 LD_{50} concentration, injections being given at hourly intervals. This same dose of toxin was also given in later experiments (about half of the total number) as 0.21 cc of a 20 LD_{50} concentration. For the intraperitoneal route, the LD_{100} was 5.0 LD_{50} when given in 3 divided doses. The lipids to be tested were also given either in 3 divided doses intravenously or intraperitoneally, or in a single dose by either route. The precaution of dividing the doses of toxin and of lipids was first adopted in order to lessen the vascular concentrations at a single moment. The survival rate of mice given toxin and lipids was slightly higher where this precaution was taken, but not sufficiently so to justify the extra work involved. In Table III results from both methods of injection have been grouped together. In all cases, the lipid was given one to 10 minutes prior to the toxin, by means of a separate needle and syringe. The dosages of lipids ranged from 0.3 to 1 g per kilo, administered as a 2 to 10% emulsion, and the volume of fluid injected ranged from 0.1 to 0.5 cc per injection.

Results. Inhibitory action of lipid prepara-

TABLE I.

Inhibitory Action of Certain Substances on *in vitro* Hemolysis by *Cl. welchii* Toxin.

	%
Total lipid extract from human erythrocytes	0.1
Egg lecithin*	0.1
Calf brain phosphatidyl serine	0.1
Soy bean phosphatides†	0.1
Oleyl stearyl-diglyceride‡	0.1
Phosphorylcholine§	0.2
Sodium glycocholate	0.8

* Eastman Kodak Company.

† Obtained through the courtesy of the American Lecithin Company.

‡ Obtained through the courtesy of Lever Brothers.

§ Obtained through the courtesy of Dr. Gerhardt Schmidt.

11 Folch, J., and Van Slyke, D. D., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 514.

12 Shohl, A. T., *J. Lab. and Clin. Med.*, 1940, **25**, 1325.

TABLE II.
Experiments on Protection of Mice Against *Cl. welchii* Toxin by Intraperitoneal Injection of Lipid Preparations Administered by Separate Intraperitoneal Injection.

Type of material	Survival rate: Lipids and toxin	Survival rate: Toxin alone
Human erythrocyte total lipids	13 out of 13	1 out of 11
Egg yolk total lipids	7 " " 8	2 " " 6
" " lecithin*	16 " " 16	1 " " 18
Soy bean phosphatides	0 " " 6	0 " " 4
Phosphatidyl serine[13]	0 " " 4	0 " " 2
Human erythrocyte total lipids I-V, and toxin given I-P	0 " " 4	0 " " 2

* Eastman Kodak Company.

tions and other substances on in vitro *hemolysis by* Cl. welchii *(type A) toxin:*

A number of lipid preparations and other compounds were tested for inhibitory action on hemolysis. They all showed inhibitory powers. The substances tested and the respective concentrations at which they prevented hemolysis completely are listed in Table I.

Experiments on mice: Tolerance for intraperitoneal or intravenous injection of lipid preparations:

Preparations listed in Table II were well tolerated on intraperitoneal injection. Preparations listed in Tables III and IV were well tolerated on intravenous injection with the exception of pure total hog brain lipids and of sphingomyelin; the injection of either was followed by death of the mice within a few minutes. Relatively large doses (150 mg) of hog liver, hog erythrocyte, and human erythrocyte lipids were well tolerated by mice intravenously. This quantity of lipid is 15 times that necessary to provide a protective effect.

Effect of mixing toxin and lecithin in advance and injecting the mixture: When a 10% emulsion of lecithin was mixed with toxin in a test tube for a few minutes at room temperature, and the mixture, containing 4.2 LD_{50} of toxin and 75 mg of lecithin, was given by 3 closely spaced intravenous injections, 5 out of 5 mice survived, in contrast to none out of 3 controls. An effect similar to this was described by Myers[3] as a result of mixing olive oil and gum acacia with *Cl. welchii* toxin prior to administration of the mixture subcutaneously into guinea pigs.

Protection of mice, against Cl. welchii *(type A) toxin injected intraperitoneally, by separate injection of certain lipid preparations:*

Results are summarized in Table II. The minimal protective dose of lipids given by this route was approximately 7 mg.

Protection of mice, against Cl. welchii *(type A) toxin injected intravenously, by separate intravenous injection of certain lipid preparations:*

Results are reported in Table III. The difference in protective power between rat brain lipids and hog brain lipids is most likely due to the toxicity of the latter as reported above. Human erythrocyte total lipids given intravenously have not protected against the effect of toxin given intraperitoneally (Table II). When the toxin was given first intravenously and the lipids were given intravenously 15 minutes thereafter, the survival rate of the mice was cut down greatly. The minimal protective dose of total lipids (erythrocyte or liver) administered intravenously was approximately 10 mg, an order of magnitude 5 times that of the total lipids present in the red cells of a 20 g mouse.

Death of mice as a result of the toxin was associated with intravascular hemolysis, congestion and hemorrhage of the lungs, congestion of the viscera, and the presence of hemolyzed fluid in the pleural and peritoneal cavities. In agreement with the findings of Cooke, Frazer, *et al.*[16,17] fat stains showed a

[13] Folch, J., *J. Biol. Chem.*, 1941, **139**, 973.

[16] Cooke, W. T., Frazer, A. C., Peeney, A. L. P., Govan, A. D. T., Barling, S. G., Thomas, G., Leather, J. B., Elkes, J. J., and Scott Mason, R. P., *Lancet*, 1945, **1**, 487.

[17] Frazer, A. C., Elkes, J. J., Sammons, H. G., Govan, A. D. T., and Cooke, W. T., *Lancet*, 1945, **1**, 457.

TABLE III.
Experiments on Protection of Mice, Against *Cl. welchii* Toxin Injection, by Lipid Preparations Administered by Separate Intravenous Injection.

Preparation used	Survival rate: Lipid and toxin	Survival rate: Toxin alone
A. Exhibiting protective effect:		
Human erythrocyte total lipids	44 out of 53	0 out of 28
Hog erythrocyte total lipids	9 " " 13	0 " " 5
Hog liver total lipids	29 " " 34	1 " " 25
Rat liver total lipids	3 " " 3	0 " " 4
Human plasma total lipids	10 " " 14	0 " " 8
B. Failing to protect, or exhibiting questionable protection:		
Rat brain total lipids	4 " " 12	0 " " 6
Hog brain total lipids without phosphatidyl serine*	0 " " 6	0 " " 5
Hog brain phosphatides:		
Lecithin fraction	1 " " 3	0 " " 4
Cephalin fraction	4 " " 14	0 " " 6
Sphingomyelin fraction	0 " " 2	0 " " 1
Egg yolk lecithin†	0 " " 8	1 " " 13
" " total lipids	0 " " 6	0 " " 5
" " phosphatides:		
Lecithin fraction	0 " " 5	0 " " 2
Cephalin fraction	0 " " 2	
Soy bean phosphatides‡	0 " " 4	0 " " 2
Phosphatidyl serine	0 " " 6	0 " " 2
Cocoanut oil in soy bean phosphatide emulsion	0 " " 4	0 " " 2
Diglycerides in soy bean phosphatide emulsion§	1 " " 7	0 " " 4
Phosphorylcholine¶	0 " " 6	0 " " 10
Cholesterol-cephalin emulsion¶	0 " " 3	0 " " 2
Cholesterol-monoglyceride emulsion	0 " " 3	0 " " 2

* Total lipid extract prepared by the use of $Fe(OH)_3$. This method yields an extract that contains all brain lipids free of impurities and of phosphatidyl serine.[14]

† Eastman Kodak Company.

‡ Purified oil-free phosphatides supplied through the courtesy of the American Lecithin Company.

§ Diglycerides and monoglycerides supplied through the courtesy of the Lever Brothers Company. The emulsions were made by means of a Junior 125 Viscolizer through the courtesy of Dr. J. McKibbon.

¶ Supplied through the kindness of Dr. Gerhardt Schmidt. Administered 0.25 cc of a 2% solution.

¶¶ Prepared according to the technic of Hanger.[15]

minor degree of lipid embolism of the lungs and lipid staining material in the spleen. Sections taken from mice, protected for 24 hours against the toxin by injection of liver lipids, were fixed in 10% formalin, and frozen sections therefrom were stained with Sudan IV. No evidence of lipid embolism was found in the lungs, but the livers showed a slight increase in the amount of fat staining material.

Effect of fractionation on the protective action of total lipid extracts of hog liver: During the fractionation, it has been found that the lipid preparation must not be exposed to air or light at room temperature for more than the shortest possible length of time. In two preliminary experiments done on a small scale, in which such precautions were not strictly adhered to, the result was the disappearance of the protective effect of the lipid preparations involved. The purified total lipids appear to be less sensitive to such changes than the fractions obtained therefrom; the protective effect of aqueous emulsions of purified total lipid preparations remained present for a number of days when they were simply kept at ice-box temperatures.

In a third and fourth fractionation experiment, each performed on 15 g of purified total hog lipids the fractions were tested with re-

[14] Folch, J., *J. Biol. Chem.*, 1942, **146**, 35.

[15] Hanger, F. M., *J. Clin. Invest.*, 1939, **18**, 261.

TABLE IV.
Fractionation of Total Hog Liver Lipids. Protective Effect of Fractions Against *Cl. welchii* Toxin in Mice. Route of Injection Intravenous.

Type of material injected	Survival rate
Toxin alone	0 out of 22
Total liver lipids and toxin	18 " " 21
Fractions and toxin:	
Acetone soluble (fat and cholesterol)	2 " " 13
Alcohol soluble (lecithin)	10 " " 16
Alcohol insoluble (cephalin)	0 " " 13
Recombined fractions	11 " " 13

sults summarized in Table IV. The protective action appears to reside with the acetone-insoluble, alcohol-soluble fraction, the fraction presumably richest in lecithin. The slight protective action exhibited by the acetone-soluble fraction is possibly due to contaminating lecithin.

Effect of lipids on development of immunity to Cl. welchii *toxin:* Of a group of 19 mice surviving a lethal dosage of *Cl. welchii* toxin as a result of lipid injection, 10 mice survived a repeat lethal dose (4.2 LD_{50}) 10 days later. Two weeks after survival, 2 dogs listed in Table V were tested and found to have a good serum antitoxin titre against *Cl. welchii.*

Experiments on dogs: Two dogs were given 220 LD_{50} per kilo of *Cl. welchii* toxin by slow intravenous drip into the right femoral vein over a 3 hour period. Over a 4 hour period, beginning one hour prior to the administration of the toxin, a 10% aqueous emulsion of human erythrocyte total lipids was administered by slow intravenous drip into the left femoral vein. (1.6 g per kilo for one dog and 1.1 g per kilo for the other dog). These dogs were under sodium pentobarbital anesthesia (30 mg per kilo), with carotid and tracheal cannulation for measurement of blood pressure and cardiac output. Two control dogs given this dosage of toxin alone under the same experimental conditions died with extensive intravascular hemolysis within 8 hours after the beginning of toxin administration. Autopsies revealed the presence of hemolyzed fluid in the pleural and peritoneal cavities, extensive pulmonary edema and hemorrhage, and congestion of the viscera. At the end of the same length of time, the 2 dogs given erythrocyte lipids in addition to toxin were in good condition. Throughout the course of the experiment, their plasmas showed minimal hemolysis (under 0.1%) with unchanged hematocrits, and their cardiac outputs remained within normal limits. These 2 dogs were sacrificed after 8-10 hours, and the autopsies were negative except for mild pulmonary congestion in one animal and moderate congestion of the liver in the other. One of the dogs showed a temporary marked fall in blood pressure when the rate of administration of lipids exceeded 10 cc per minute.

The plasma concentration of total lipids in the dog given 1.6 g per kilo rose from a control level of 0.6 g % to 2.0 g % at the end of the lipid injection, then declined during the subsequent 5 hours to 1.3 g %. In the dog given 1.1 g total lipids per kilo, the plasma lipid level rose from a control level of 0.6 g % to 1.5 g % at the end of the injection, then declined over the subsequent 4 hours to 1.1 g %.

One unanesthetized dog was first given 1.3 g per kilo of a 3.8% (in 0.9% sodium chlor-

TABLE V.
Dog Protection Experiments.
(Route of injections intravenous. All dogs received 220 LD_{50} *Cl. welchii* toxin per kilo body weight.)

Total lipid dose (g per kilo)	Result
1.6*	Survived
1.3	"
1.1	"
1.1*	"
0.4	"
0.4	Died
0	"
0	"
0	"
0	"

* Human erythrocyte total lipids. The others were hog liver total lipids.

ide) emulsion of hog liver lipids, and then was given 220 LD_{50} per kilo of toxin, and survived. Two unanesthetized dogs were given 0.4 g per kilo of a 2% emulsion of hog liver total lipids. Half of the dose was administered by syringe prior to intravenous injection of toxin (220 LD_{50} per kilo) and the other half after the toxin. One dog survived, and the other died. The results of the dog experiments are summarized in Table V. In these unanesthetized dogs, the rather large volumes of lipid (100-200 cc) injected were administered rapidly in 2 or 3 50-75 cc doses, and were well tolerated. The precaution of adjusting the pH of the emulsion above 7.0 prior to injection was observed in these experiments.

Discussion. A number of substances (Table I) have been found by the authors to inhibit the hemolysis of erythrocytes *in vitro* by *Cl. welchii* toxin. Only lipid preparations hydrolyzable by *Cl. welchii* toxin, however, have been able to protect mice and dogs against *Cl. welchii* toxin given by separate injection. Cholesterol has for a long time been known to exert a protective action against red cell hemolysis *in vitro*;[18,5] but as indicated in Tables III and IV, it has little protective effect against *Cl. welchii in vivo*. It is possible that the presence of cholesterol enhances the protective effect of lecithin as it exists in the total lipid preparations.

The fact that the split products of the hydrolysis of lecithin by the *Cl. welchii* alpha toxin are relatively non-toxic is an important consideration. In contrast to this situation is the production of the highly toxic, hemolytic lysolecithin by cobra venom lecithinase in its action on lecithin.[19] Injection of lipids containing lecithin did not protect mice against the effects of the latter toxin, but rather appeared to aggravate the effect of the toxin.§

There is no explanation of the inability of fresh egg yolk total lipids to protect mice on intravenous injection. As stated previously, purified preparations of lecithin quickly lose their protective effect on exposure to light and air, whereas, purified total lipids retain their protective effect for a number of days if they are simply kept as aqueous emulsions at 3° C.

It should be mentioned that *Cl. welchii* (type A) toxin as it occurs in war wounds and as prepared by Dr. Milan A. Logan, contains small amounts of theta toxin and a considerable amount of hyaluronidase, against which the lipids presumably have no protective effect.

The usefulness of this approach to the problem of *Cl. welchii* infection is limited by the lack of ability of the lipids to reverse damage to cells which has already occurred, as a result of toxin, prior to their administration. It is worth noting, however, that in experiments herein reported the concentrations of toxin introduced into the blood stream at a single instant far exceed those which might be expected in clinical *Cl. welchii* toxemia.

When knowledge of the substrate specificity of other bacterial toxins becomes available, it should be possible to apply a similar chemotherapeutic approach to other cases. This type of approach can theoretically be best used where the enzyme can be "saturated" with substrate (*i.e.* where $V=K_2E$, according to Van Slyke[20]).

Summary. Purified total lipids obtained from erythrocytes, plasma, and liver have been found to have a protective effect against *Cl. welchii* (type A) toxin in mice and dogs. The protection appears to be due to a substrate partition effect in which the lecithinase of the *Cl. welchii* toxin hydrolyzes the relatively large added source of lecithin and in part spares the lecithin of the animal cells from destruction.

18 Theorell, H., *Biochem. Z.*, 1930, **223**, 1.

§ The authors are indebted to Hynson, Westcott, and Dunning, Inc., for a supply of dried cobra venom.

19 Levene, P. A., Rolf, I. P., and Simms, H. S., *J. Biol. Chem.*, 1924, **58**, 859.

20 Van Slyke, D. D., in Nord, F. F., and Werkman, C. H., *Advances in Enzymology and Related Subjects*, New York, 1942, **2**, 33.

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15086

Influence of Dosage and Volume on the Circulation Time.

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While using the subjective nut-like taste and smell which follows the intravenous injection of thiamine as a method of determining the circulation time,[1] we found that our figures for normal individuals ranged from 5 to 12 seconds, the great majority being between 7 and 9 seconds. Other subjective methods involving taste (Table I) give variable but, in general, higher figures, according to the literature. This is also true of most of the so-called objective methods (Table I), involving detection of radioactive substances, dyes, or deep inspiration caused by stimulation of the carotid sinus or the respiratory center in the medulla. The papaverine method of Elek and Solarz,[2] presumably involving the stimulation of the medullary respiratory center, gives figures for normals of 15.9 to 27 seconds, which seem to be much too long. On the other hand, the improved lobeline method, utilizing the perception of "tickling" in the throat as well as carotid sinus stimulation of respiration, in the hands of Piccione and Boyd[3] gave results in normals entirely comparable to ours, 5 to 12 seconds. The results would have been somewhat higher had they included the time of injection. Bernstein and Simkins[4] stated that 7-17.8 seconds were the normal circulation times for their routine dose, 6 cc of 10% magnesium sulphate, but mentioned that in "sensitive patients" doses of as little as 2 cc gave circulation times as low as 6 seconds. We have obtained similar low values in some normal individuals, especially with one gram doses of magnesium sulphate as well as the usual doses of intravenous arsenicals used in the treatment of syphilis.[5] Different points of detection alone cannot explain the marked differences in circulation times obtained by the various methods in comparable patients (Table I), or the same patients.[1–6] It must be remembered, however, that the velocity of blood flow is considerably greater in the arteries than in the arterioles, capillaries and venules, and that the latter channels are perhaps variably involved in all of the above methods.

The diodrast method of Robb and Steinberg[7] for visualization of the heart and great vessels may be considered an objective method for the determination of circulation time. The left ventricle has been visualized by the authors in 6 to 9 seconds, and sometimes longer. If approximately 1 second is added for further transmission to the tongue,[8] the resulting circulation time is closer to our figures than to most other published in the literature (Table I), including the authors' own sodium cyanide circulation times. Because this method employs large volumes (35 cc), injected under pressure through a large bore needle, with the arm held up and the body vertical, it was felt necessary to study the effect of varying volumes, and of the position of the body and arm, especially since it is generally assumed that large volumes give too high a reading because of the delay in injection. As early as 1922, Loevenhart

1 Ruskin, A., and Decherd, G., *Federation Proc.*, 1945, **4**, 62.

2 Elek, S. R., and Solarz, S. D., *Am. Heart J.*, 1942, **24**, 281.

3 Piccione, F. V., and Boyd, L. J., *J. Lab. and Clin. Med.*, 1941, **26**, 766.

4 Bernstein, M., and Simkins, S., *Am. Heart J.*, 1939, **17**, 218.

5 Ruskin, A., Rockwell, P., and Decherd, G., unpublished observations.

6 Hussey, H. H., Cyr, D. P., and Katz, S., *Am. Int. Med.*, 1942, **17**, 849.

7 Robb, G. P., and Steinberg, I., *Am. J. Roentgenol.*, 1939, **41**, 1.

8 Blumgart, H. L., and Weiss, S., *J. Clin. Invest.*, 1927, **4**, 15.

TABLE I.
Normal Human Circulation Times by Various Methods.

Substance	Effect at	Circ. time (sec.)	References
Radium-C	other arm	12 -23	Blumgart and Weiss, 1927
Thorium-X	" "	10 -20	Gerlach *et al.*, 1943
Radioactive sodium	" " (children)	5 -17	Hubbard *et al.*, 1942
Fluorescein (ultra violet)	conjunctiva	7 -15.6	Fishback, 1941
	lip	15 -20	Lange and Boyd, 1942
Methylene blue (photoelect.)	skin	7 -21.6	Jablons *et al.*, 1942
Lobeline	carotid sinus	5 -12	Piccione and Boyd, 1941
Sodium cyanide	" "	9 -21	Robb and Weiss, 1933
Aminophylline	medulla	7.1-20.4	Koster and Sarnoff, 1943
Papaverine	medulla?	15.4-27	Elek and Solarz, 1942
Histamine	skin	13 -30	Weiss *et al.*, 1929
Decholin	tongue	10 -16	Tarr *et al.*, 1933
Saccharin	"	9 -16	Fishberg *et al.*, 1935
Calcium chloride	"	9 -15	Kahler, 1930
Calcium gluconate	"	10 -16	Goldberg, 1936
Magnesium sulphate	"	7 -17.8	Bernstein and Simkins, 1939
Magnes. Sulph. comp. (Macasol)	"	5 -24	Kvale and Allen, 1939
Diodrast	left ventricle	6 - 9	Robb and Steinberg, 1938
Thiamine	tongue and nose	5 -12	Ruskin *et al.*, 1945

et al.,[9] using sodium cyanide for the determination of circulation time in animals, found that sub-optimal doses gave no, or false high, readings. We[5] have noted the same effect in patients, particularly those in cardiac failure, when employing either magnesium sulphate or thiamine. It seemed likely that variation in volume, dosage, and possibly position of the patient might explain in part the highly variable data thus far obtained by different workers.

Method. The effect of dosage was determined by injecting thiamine hydrochloride* in doses from 5 to 900 mg (0.1 cc to 3 cc, diluted as needed), magnesium sulphate 0.05 to 1.0 g (0.1 to 2 cc of the 50% solution), sodium dehydrocholate (decholin) 0.05 to 1.0 g (0.25 to 5 cc of the 20% solution, diluted as needed), and theophylline-ethylenediamine (aminophylline) 0.01 to 0.48 g (0.05 to 2 cc of the 24% solution). Various doses of the same drug were injected in the same patients, using those confined to bed but without demonstrable heart disease, on different days, in approximately the same near-basal condition, either before breakfast, or during the day before meals. Our observations agree with those of other workers,[10–13] that no perceptible error is thus introduced. Rapid injection into the antecubital vein required 2 seconds or less, with the stop watch timed from the beginning of the injection. The majority of the observations were done in duplicate and the curves (Fig. 1-5) were drawn from their averages.

The effects of volume, pressure and posture, were studied by using thiamine hydrochloride in a constant dose of 300 mg, a supra-optimal dose for the 5 patients studied (Fig. 6). This amount, diluted with normal saline solution to volumes varying from 1 to 30 cc, was injected through a 14 gauge needle. In one instance (patient C.F.), the dose was injected repeatedly in 1 and 30 cc volumes on different days with the results shown. In 2 other instances the 1 and 20 cc volumes were injected in the antecubital vein in the direction opposite the venous flow as well as in the same direction (Fig. 6). In 2 other instances 20 cc volumes were also injected with the patient standing upright and the arm elevated as in the clinical diodrast method.

Results. Effect of Dosage. When the circu-

[9] Loevenhart, A. S., Schlomovitz, B. H., and Seybold, E. G., *J. Pharm. and Exp. Therap.*, 1922, **19**, 221.

* Kindly supplied to us by Dr. H. S. Newcomer, E. R. Squibb and Sons, in 10 cc rubber-capped vials, containing exactly 300 mg per cc.

[10] Reingold, I. M., Neuwelt, F., and Necheles, H., *J. Lab. and Clin. Med.*, 1942, **28**, 289.

[11] Tarr, L., Oppenheimer, B. S., and Sager, R. V., *Am. Heart J.*, 1933, **8**, 766.

[12] Goldberg, S. G., *Am. J. Med. Sc.*, 1936, **192**, 36.

[13] Kvale, W. F., and Allen, E. V., *Am. Heart J.*, 1939, **18**, 519.

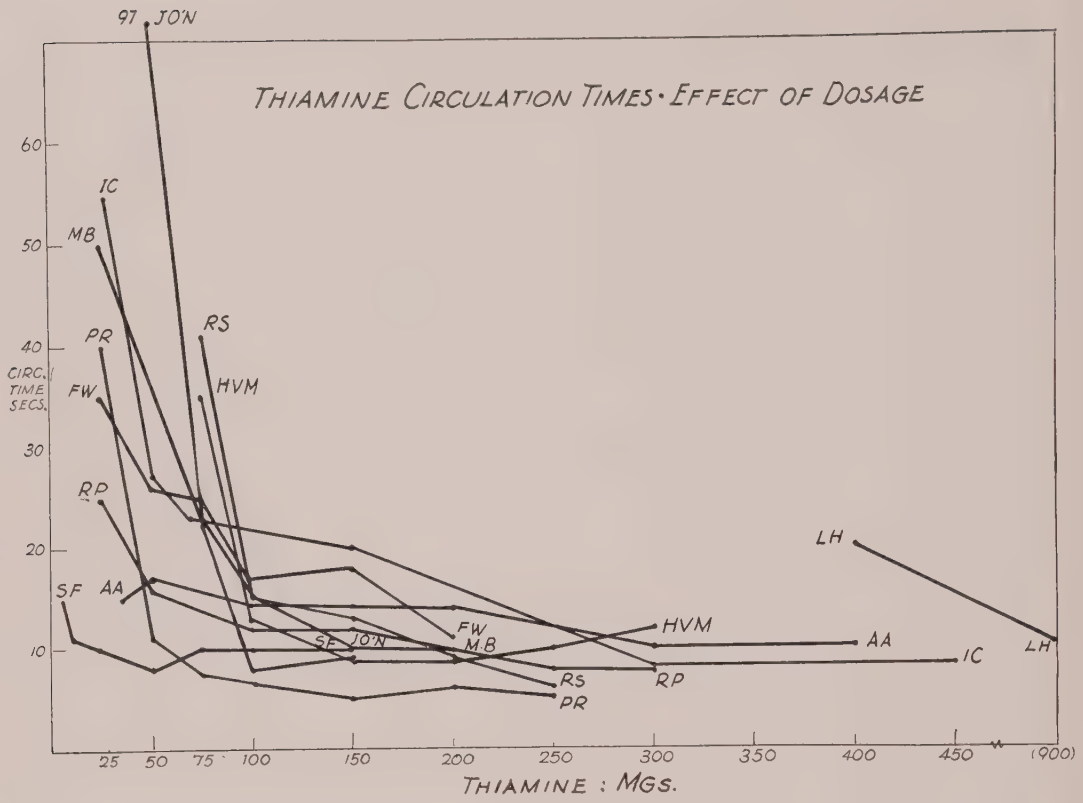

FIG. 1.

Effect of various dosages of thiamine hydrochloride upon the circulation times of 11 patients free from demonstrable heart disease.

lation time in seconds was plotted against dosage, logarithmic curves were obtained in most instances. (Fig. 1-5). For thiamine low doses gave rather higher readings than for decholin and magnesium sulphate; aminophylline showed the narrowest span of circulation times for varying doses. It will be seen that the lowest dose of any drug first producing a response varied in different individuals. Likewise the optimal dose reaching the asymptotic level, or producing the shortest circulation time, varied similarly. Thus, one individual (S.F., Fig. 1) could taste and smell thiamine with a dose as low as 5 mg, and 25 mg was his optimal dose, higher doses not shortening the circulation time appreciably. Another (L.H. Fig. 1) gave no response until 400 mg was injected and gave a much lower reading of the circulation time with 900 mg. This patient was a rare exception, who with a standard dose of 300 mg would have been classified as a nonreactor. The asymptotic or optimal doses obtained were, in general at least 100-300 mg for thiamin, 0.24-0.48 g for aminophylline, 1.0 g for magnesium sulphate, and 1.0 g for decholin. This would indicate that dosages previously used in investigative and clinical work may have been too low and, hence, the readings so obtained too high.

Effect of Volume and Position. Fig. 6 shows the effect of volume on the circulation time, and it will be seen that when 300 mg of thiamine was injected in 5 cc of solution instead of 1 cc the circulation time was shortened by 1-2 seconds. The 20 cc and 30 cc volumes, as compared to 1 cc, shortened it by 3-5 seconds. 1 cc volumes injected up or down the vein made no difference in circulation time in two instances. Twenty cc volumes, however, injected in a direction opposite the venous flow, actually lengthened the circulation time by 1-2 seconds. Whether the 20 cc volumes were injected in horizontal or

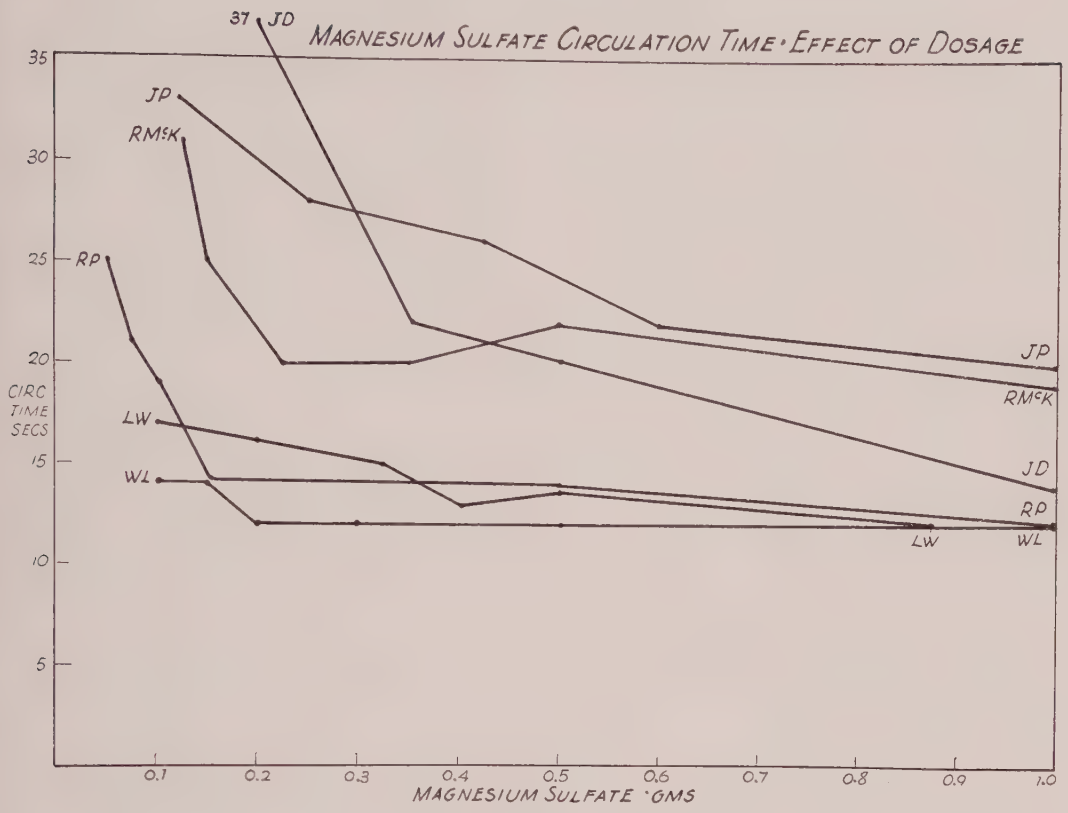

FIG. 2.

Effect of various dosages of magnesium sulphate upon circulation times of 6 patients, free from demonstrable heart disease.

vertical body and arm positions made no difference in circulation times.

Comment. The optimal dose of any agent used for determining the circulation time is that dose which gives values in the asymptotic portion of the curve (Fig. 1-5). Our study shows that the optimal dosage of at least 4 drugs used in the measurement of circulation time varies with the individual and the drug; that low or suboptimal doses give no end-points or end-points that are too high, and that these also vary with the individual and the drug. It follows that in any study, clinical or experimental, the use of a standard dosage will result in some failures to obtain an end-point, and this is probably true, contrary to some claims[2,4,11,12,14] of almost any drug in clinical usage.[1,3,5,6] It also follows that the wide scatter and high range of "normal" circulation times by various methods may be due to disregard of the logarithmic variation of circulation time with dosage. This is even more true of patients in congestive heart failure.[4,5] Failures or unduly high and variable readings are also obtained especially for distant points of detection, such as the feet, hands and perineum.[5,13] In other words, either slowing of the circulation as in congestive heart failure, or greater distance to the points of detection result in dilution, or, in effect, lower the amount of the drug actually reaching the place of detection; the end result is that of a suboptimal dose. In congestive heart failure a higher threshold of stimulation or perception may also be present and different optimal doses for the estimation of circulation times in various stages of heart failure may be required for this reason as well. We have attempted[5] to study the effect of dosage in patients with congestive heart failure, and the results have

[14] Robb, G. P., and Weiss, S., *Am. Heart J.*, 1933, **8**, 650.

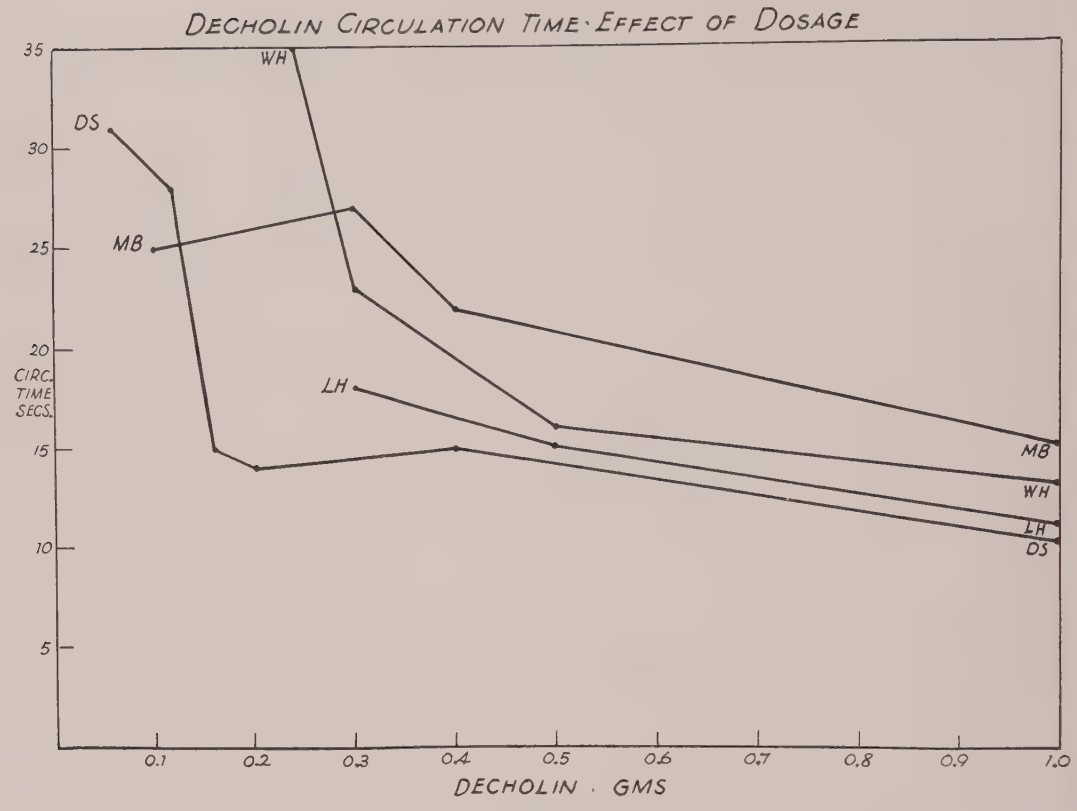

FIG. 3.

Effect of various dosages of decholin upon the circulation times of 4 patients, free from demonstrable heart disease.

shown the same trends as in the present study, but have been too variable for graphic representation.

Our findings are in essential agreement with the evidence obtained in animals by Loevenhart *et al.*[9] of prolonged circulation times when subminimal doses of sodium cyanide were used. Robb and Weiss[14] likewise stated that relatively low doses of sodium cyanide gave circulation times in patients from 3 to 10 seconds longer than those obtained from larger doses, and presented a case showing a reduction of the circulation time from 22 to 16 seconds upon increasing the dose from 5 to 10 mg. Elek and Solarz[2] likewise tried the effect of increasing the dosage of papaverine from 32 to 56 mg in 2 cases, with corresponding reduction of the circulation time from 25 to 21 seconds, which they interpreted, however, to be of no significance.

It would be of great interest to determine whether the same logarithmic curves hold for varied dosage of radioactive substances and dyes now used (Table I) as objective measurements of circulation time. We have noted that the trend holds for one objective method —the aminophyllin one, and we have mentioned above that two groups of workers pointed it out for another one—the sodium cyanide method.

In a review of the subject Blumgart[15] defined circulation time as the interval of time necessary for the fastest particle of a foreign substance to traverse the shortest possible path between the point of injection and the place of detection. Our experiments indicate that it takes not one but a certain concentrated number of particles per unit time to be detected by the taste buds of the tongue, or the sensory nerves of the throat, or to stimulate the respiratory center enough to produce an easily detectable inspiratory gasp.

15 Blumgart, H. L., *Medicine*, 1931, **10**, 1.

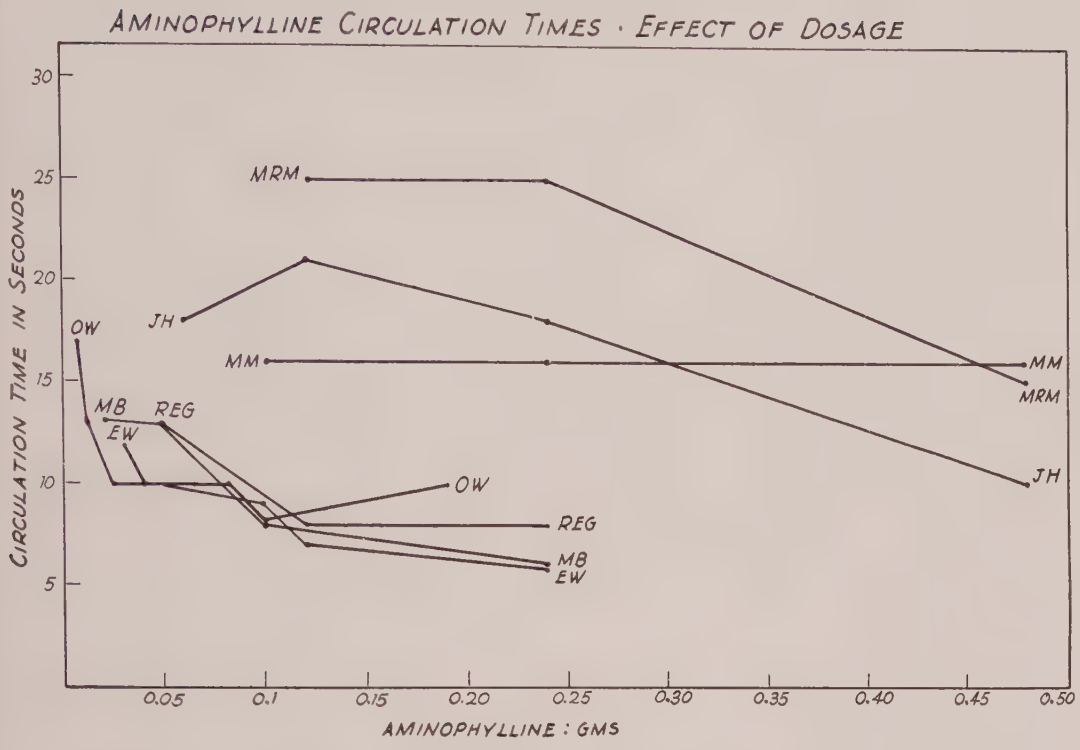

FIG. 4.

Effect of various dosages of aminophylline upon the circulation times of 7 patients free from demonstrable heart disease.

In other words, strength and duration of the stimulus have to be considered in actual practice. It was our impression, without pneumographic evidence, that suboptimal doses of aminophylline produced a poorly defined and more gradual as well as late inspiration, if they gave an end-point at all. With magnesium sulphate, decholin, and thiamine we have noted, too, that the degree of sensation of heat or taste, was, in general, in proportion to the dose used. Similar observations have been made for sodium cyanide,[10] lobeline,[3] and papaverine.[2] While proof is not now available, it is reasonable to believe that the same statements probably held for the detection of radioactive substances by the Geiger counter or dyes by the photoelectric cell and galvanometer, or ultraviolet light fluorescence. It was noted that suboptimal doses of aminophylline produced less lengthening of circulation time than did those of the subjectively appreciated drugs. This may be true of other objective methods, and the shorter the span of circulation time with dosage, the flatter the curve, the more dependable should that method be. On the other hand, the safest maximum dosage of the various drugs listed in Table I, with the exception of thiamine and a few others, may not give the shortest or asymptotic circulation times in individual cases. Our observations[5] indicate that the sensory nerves of the tongue and throat are apparently depressed in some patients by smoking, old age, and previous strong tastes, with resulting failure or undue prolongation of the circulation times when subjective methods involving taste or heat perception are used. Such is the frequent effect, for instance, of repeating the drug injection within several minutes of the previous test, contrary to the claims of other observers[4,11,12] that comparable duplicate measurements may be thus always obtained. A higher threshold of stimulation, thus, may act just as a suboptimal dose. This criticism may not hold for the so-called objective methods;[2,5,16] further study is necessary.

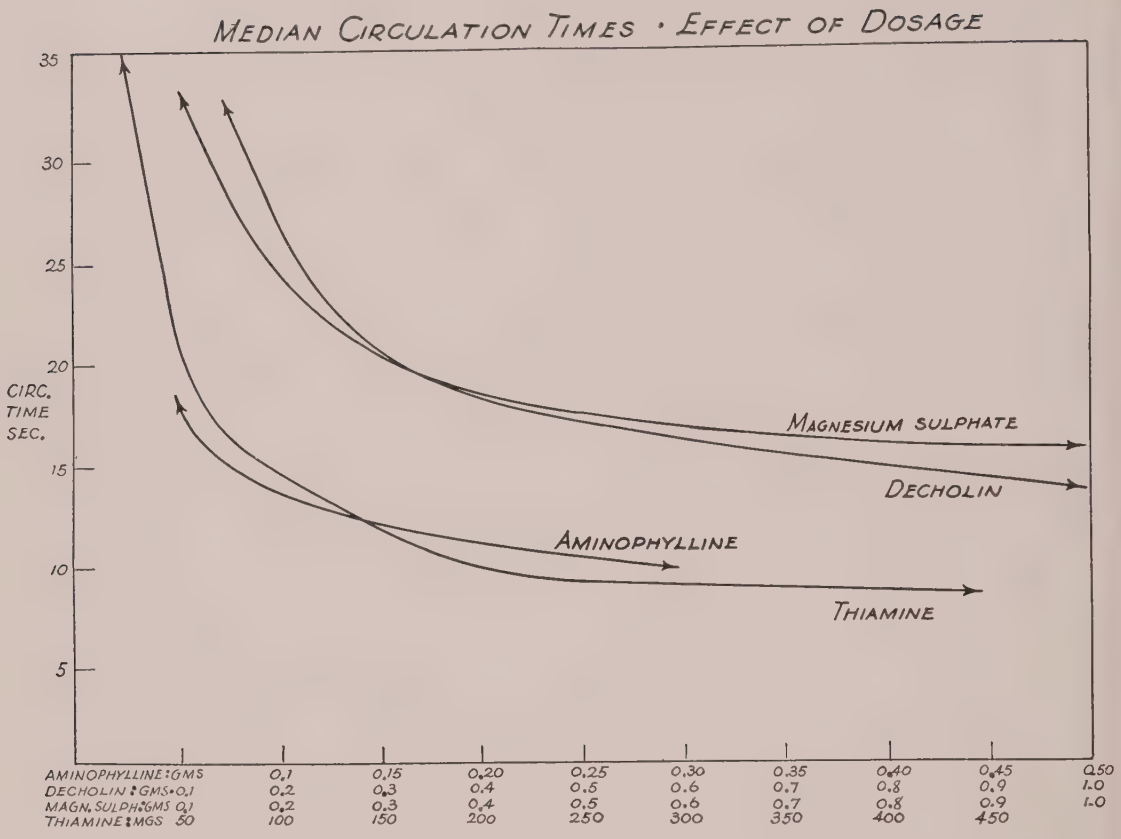

FIG. 5.

Curves showing median circulation times as dependent on dosage of thiamine, magnesium sulphate, decholin, and aminophylline.

Another limiting factor, particularly true of decholin, 10% magnesium sulphate or calcium salts, and especially, diodrast, is volume of solution. Loevenhart *et al.*[9] showed for sodium cyanide in animals, and most subsequent investigators implied, that duration of injection over one second will prolong the circulation time. Efforts had been made, therefore, to limit the volume of solution and inject it as rapidly as possible, if necessary through a large bore needle. Winternitz *et al.*[17] noted, without explanation, that dilution of the dose of histamine recommended by Weiss *et al.*,[18] to 5 cc gave shorter and more constant circulation times than had been reported by the latter group. The results of Winternitz's group are not comparable, however, with those of other observers because their measurements were begun at the end, rather than at the beginning, of injection. Our results show that volumes of 5 cc shorten the thiamine circulation time by 1-2 seconds, and volumes of 20-30 cc by 3-5 seconds. Preliminary observations indicate that this is apparently also true of other methods.[5] Other variable factors, however, are involved in this shortening. One is the great pressure exerted, which mechanically propels the larger volumes forward to the heart, since the capacity of the venous channels up to the right auricle is not much greater than the volumes of fluid injected. That pressure as well as volume is involved is also shown by the fact that injection in the direction opposite the venous flow actually lengthens circulation time despite the over-

[16] Koster, H., and Sarnoff, S. J., *J. Lab. and Clin. Med.*, 1943, **28**, 812.

[17] Winternitz, M., Deutsch, G., and Brüll, Z., *Med. Klinik.*, 1931, **27**, 986.

[18] Weiss, S., Blumgart, H. L., and Robb, G. P., *Am. Heart J.*, 1929, **4**, 664.

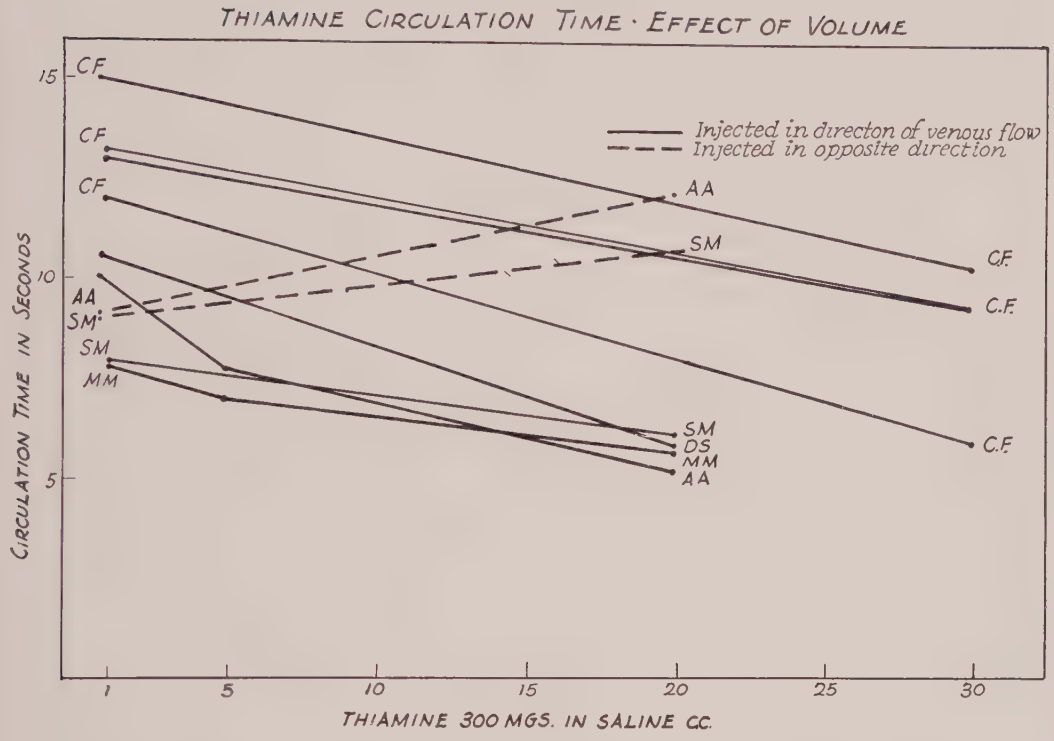

FIG. 6.

Effect of injection of 300 mg of thiamine hydrochloride in volumes of saline varying from 1 to 30 cc on the circulation times. Dotted lines represent effect of injecting the various volumes in patients A.A. and S.M. in the direction opposite the venous flow.

filling of the veins. We have not attempted to study the effects of quantitated pressures. Despite the slowness of injection of the larger volumes in some cases, up to 3-4 and even 5 seconds, the circulation time, measured from the beginning of injection was always shortened. In other words, the effect of dilution of the drug by slow injection, which has been shown to act in opposite fashion,[9] was counterbalanced by the factors of volume and pressure. Other factors, such as the effects of streamline versus turbulent flow, the former of which is known to double the speed of flow in the central portion of the stream as compared with average velocity,[19] are impossible to evaluate from our data.

19 Vennard, J. K., *Elementary Fluid Mechanics*, 1940, p. 113, John Wiley & Sons.

Summary. 1. By the use of various dosages of thiamine, magnesium sulphate, decholin and aminophylline, it was shown that lower doses are either ineffective or produce prolonged circulation times. 2. Logarithmic curves express this relationship of circulation time to dosage. These vary from person to person. 3. Beyond a certain dose, varying in different individuals, further increase in dosage causes no appreciable decrease in circulation time. This may be called the optimal or asymptotic dose, and the time the shortest or "true" circulation time. 4. Increasing the volumes containing the same doses of thiamine to 5 cc or more, shorten the circulation times markedly enough to affect clinical measurements.

15087

Certain Affections of the Liver That Arise Spontaneously in So-Called Normal Stock Albino Mice.*

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During the course of attempts to find an agent possibly present in human infectious hepatitis, certain spontaneous lesions were encountered in the liver of "normal" stock albino mice which could confuse workers in this field.

The present paper reports on the pathological changes observed in young and old mice used in this investigation. Another phase of the problem, the application of the thymol turbidity test[1] for detection of hepatic dysfunction in the mouse, is still being studied and the results will be described in another article.

The disclosure of the hepatic lesions came about in this way: Serum or stool deriving from patients in the acute phase of infectious hepatitis was prepared as filtrates, ultracentrifugates, or left unchanged, and the material was injected into mice of the Swiss strain by a variety of routes. The mice generally (with few insignificant exceptions) showed no signs of disturbed health for periods of observation lasting beyond 5 months. Certain ones were sacrificed at different intervals beginning at 2 weeks after injection and continuing at 1 to 2 weeks' intervals for 3 months or longer after inoculation. When it was found that the liver of every animal exhibited changes no matter whether it had received test or control material, suspicion was aroused that the pathological reaction was nonspecific and a study was therefore undertaken based on the histopathology of the organ of normal mice of the Swiss as well as of the Rockefeller Institute strain.†

Two affections of the liver have been encountered in so-called normal stock mice. Whether they are different aspects of the same affection or two pathological entities, is not known. One was characterized by infiltration and necrosis, and the other by intranuclear inclusion bodies. Both conditions were distinct from the familiar cyst formation, atrophy, congestion and secondary involvement of the liver in experimental or natural infections with various microorganisms or viruses, or other changes met with heretofore in the laboratory. In both, the gross anatomy of the liver and other organs was normal and no associated microorganism or causal agent has as yet been recovered. To all appearances, animals bearing the lesions have been in good health.

Infiltrative and Necrotic Lesions. (Fig. 1 and 2). This type is commonly present in both strains of mice to varying degrees and more pronounced with increasing age, up to 1 year or older. Nurslings as young as 6 days exhibited the changes in all of 6 examined.

The histopathology consists of an infiltration, collar-like, chiefly about the smaller bile ducts, sometimes also about blood vessels, of cells of which polymorphonuclear leucocytes are predominant; monocytes, lymphocytes and plasma cells are also seen. There may be a few cells in the collar, or as many as a dozen rows. No bacteria, protozoa, or other microorganisms are detectable in the infiltrated areas. Here and there a small or large focus of these cells lies by itself in the parenchyma. The capsule of the liver is not

* This study was carried out under the Commission on Neurotropic Virus Diseases, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, U. S. Army.

1 Maclagan, N. F., *Brit. J. Exp. Path.*, 1944, **25**, 234.

† Many thanks are due Dr. Charles L. Hoagland and Lieut. Commander Robert E. Shank, U.S.N.R., of the Hospital of the Rockefeller Institute, for their generous and wholehearted cooperation.

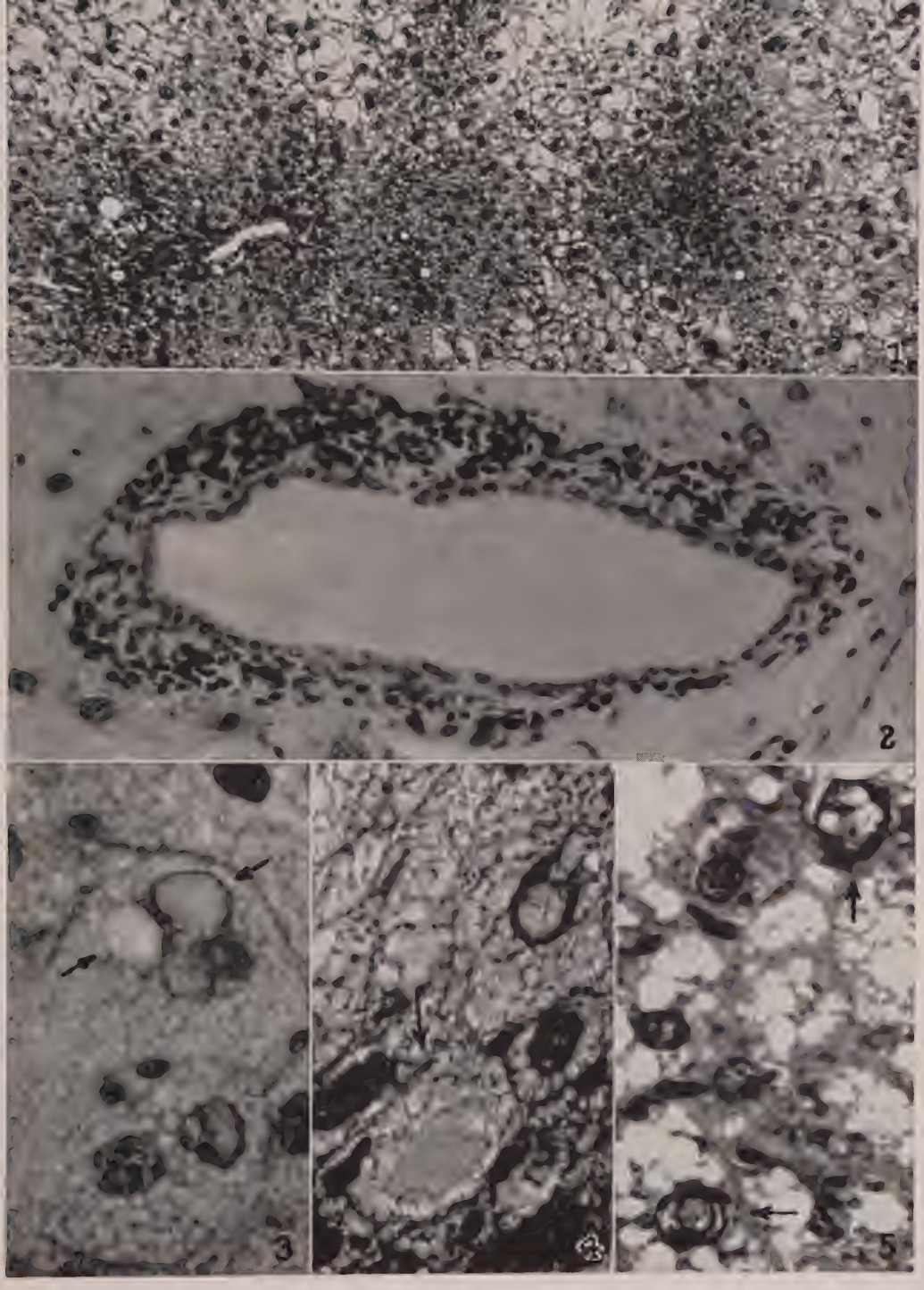

FIG. 1. Normal, 15-months-old mouse; necrosis of parenchymal cells of liver. Hematoxylin and eosin stain. × 275.

FIG. 2. *Idem.* Cellular infiltration of bile duct. Hematoxylin and eosin stain. × 425.

FIG. 3. Mouse about 3 months old, given infectious hepatitis material intranasally and examined 70 days later. To be noted are multinucleated giant hepatic cells with intranuclear inclusion bodies, and margination of nuclear chromatin. The topmost nucleus has an inclusion filling the entire nucleus, which is vacuolated, and next to it is an "empty" or "ghost" nucleus. Eosin-methylene blue stain. × 1000.

FIG. 4. *Idem.* A Councilman body (only rarely seen in these tissues) and nearby an intranuclear inclusion body surrounded by basichromatin, also margination of nuclear basichromatin. Giemsa stain. × 1000.

FIG. 5. Normal, 15-months-old mouse. Intranuclear inclusion with margination of basichromatin. Above, hypertrophied nucleus with inclusions and margination. Hematoxylin and eosin stain. × 950.

thickened; occasionally sinusoids are engorged; the architecture of the lobule is generally retained (Fig. 1). Necrosis of individual parenchymal cells is noted, or of larger zones usually about the central or about the intercalated (sublobular) veins, or sometimes midzonal. There is more variation in the degree of cellular necrosis than of infiltration. Of 97 mice examined, only 6 failed to reveal the infiltrative and 12 the necrotic or necrobiotic lesions. The liver cells lose their cytoplasmic contents, the nucleus is not visible and the cell body may disappear completely—yet the arrangement of the lobules is retained—a cell or a collection of cells becoming necrosed in its own site. Thus the necrosis is generally focal, not diffuse, and the areas are not surrounded by or involved in an inflammatory reaction or subject to autolysis. It would therefore appear that these changes are compatible with the seemingly good health of the animal and the liver can be regenerated to a normal appearance. Associated is the nonspecific nuclear degeneration in otherwise normal cells or in early stages of cellular necrosis. There may be a certain degree of chromatolysis and of margination of the chromatin with one or more nucleoli colored lavender or purple, instead of blue or blue-black, by Romanowsky-type and Giemsa, or hematoxylin-eosin dyes respectively. When a single nucleolus is present, it is often increased in size and still so stained. Such changes are not similar to true viral inclusions and are often seen in other conditions in various tissues.

Lesions Characterized by Intranuclear Inclusion Bodies (Fig. 3, 4 and 5). In older mice, from 3½ months to one year or more of age, and especially in the oldest of these, lesions occur in the liver, characterized by the presence of intranuclear inclusions which have the features of typical viral inclusion bodies. The inclusions have been noted in Swiss and Rockefeller Institute strains of mice, in one of 2 aged 3½ months and in 13 of 14 at least one year old, although they were not detected in any of 6 aged 4 months. Associated generally were the infiltrative and necrotic changes just described. At this stage it is difficult to say that the latter and the inclusion-body affection are independent manifestations—the only available evidence, *viz.*, that the inclusions have been observed to be free from the infiltrative and necrotic type and younger animals having these lesions are free, as a rule, from the inclusions, is not sufficient basis for their difference.

The bodies may occur in prodigious numbers with the majority of the hepatic cells involved but more often in an occasional cell nucleus. With eosin-methylene blue, Giemsa or hematoxylin-eosin, they stain bright pink and stand out in a nucleus in which the nucleoli are dislocated to the margin and are shrunken, and the nuclear chromatin is also marginated. The nucleus is often considerably hypertrophied. A typical picture of an involved nucleus shows a dark-bluish, wide and irregular margin enclosing a clear space in which are set one or more pink-stained bodies, smaller in size when multiple, but often when one body is present it occupies the entire space (Fig. 3, right). Other nuclei exhibit no structure whatever within their membranes (Fig. 3, left), similar to the "empty" or "ghost" nuclei seen in the liver of yellow fever[2] and in that organ following burns.[3] The inclusions do not, however, resemble those of the latter two maladies, since they are not wholly granular and often reveal a single large or several small spherical vacuoles. A single vacuole may have a well-defined, whole, regular margin (Fig. 4, upper right). The inclusions are surrounded now and again by a border of basichromatin (Fig. 5, lower arrowhead). A halo or clear zone encircles the entire inclusion body which is found, as a rule, in a cell the cytoplasmic structure of which is damaged. Certain inclusions are granular with marginated chromatin corresponding to Cowdry's Type A; others exhibit the hyaline, droplet-like appearance of Type B; a classification on this basis is not possible, moreover, because still others differ from both types.

Associated with these bodies may be seen the nonspecific nuclear changes already de-

[2] Cowdry, E. V., and Kitchen, S. F., *Am. J. Hyg.*, 1936, **23**, 55.

[3] Belt, T. H., *J. Path. and Bact.*, 1939, **48**, 493.

scribed. Rarely one encounters a Councilman lesion—acidophilic, hyaline necrosis of hepatic cells[4] (Fig. 4)—binucleated, and multinucleated giant (Fig. 3) cells. Finally, the infiltrative and necrotic lesions can, as already stated, exist together with the inclusion bodies.

Discussion. Even though the studies on the causal agent of the two described types of lesions are incomplete and the relation of one to the other is as yet to be determined, a report is made at this time in an effort to assist in the problem now being pursued by several workers on the possible transmission of human infectious hepatitis to mice.

Apart from the various pathological states which may arise in the livers of mice, mentioned in this paper, others have been reported; these have been published by Dingle in his valuable monograph.[5] In addition, Dubos[6] has recently observed in several strains of "normal" stock mice, especially in older ones, hepatitis characterized grossly by whitish, circumscribed areas scattered over the surface of liver and microscopically by areas of parenchymal necrosis infiltrated by cells surrounded by an inflammatory reaction. The causal agent of this affection has not as yet been determined by Dubos, but it is apparently wholly independent of *B. piliformis* infection of the mouse liver as described by Tyzzer.[7]

The inclusion bodies here reported conform with the three terms of the definition, stated by Cowdry,[8] of inclusions due to viral action: (a) their arrangement to represent developmental stages; (b) their characteristic appearance and their association with the ultimate death of the cell, and (c) a concomitant cellular reaction of hyperplasia, hypertrophy or necrosis. Further work is needed to determine their agreement with another definition of inclusions as viral in origin, as given by Lucas and Riser:[9] their specific association with the agent or pathological condition which produces them.

The published record on this subject is scant. Twort and Twort[10] reported no characteristic inclusion bodies in 12,000 mice employed in experiments with carcinogenic agents. On the other hand, Findlay[11] described briefly intranuclear bodies in hepatic cells in all of 25 Clacton-strain mice but in none of 60 other normal mice of 7 other strains. He was able to transmit an inclusion-body malady with positive liver tissue and thus suggested that the bodies are induced by a virus of low pathogenicity. There is a resemblance between the picture of the present inclusions and the description offered by Findlay—their identity is a matter for further study. Recently somewhat similar cellular inclusions in mice have been reported by Nicolau *et al.*[12] from Roumania. They encountered these lesions in the course of their attempts to transmit the agent of infectious hepatitis to mice. The Roumanian workers have stated that certain (undescribed) lesions can be found in livers of mice before inoculation.

Conclusion. Certain pathological states which arise spontaneously in the livers of so-called normal stock mice, especially with increasing age, have been described. Of particular interest are the hepatic lesions of (a) infiltration and necrosis, and (b) of intranuclear inclusion bodies—both occurring in Rockefeller Institute and Swiss strains of albino mice. The relation of the two types of changes is still to be studied as well as their etiology.

4 Councilman, W. T., *Report on the Etiology and Prevention of Yellow Fever,* U. S. Marine Hosp. Service, 1890, p. 151.

5 Dingle, J. H., in *Biology of the Laboratory Mouse,* G. D. Snell, Editor, Blakiston Co., Philadelphia, 1941, Chapter 12, p. 380.

6 Dubos, R. J., personal communication.

7 Tyzzer, E. E., *J. Med. Res.,* 1917, **37**, 307.

8 Cowdry, E. V., *Am. J. Clin. Path.,* 1940, **10**, 133.

9 Lucas, A. V., and Riser, W. H., *Am. J. Path.,* 1945, **21**, 435.

10 Twort, J. M., and Twort, C. C., *J. Path. and Bact.,* 1932, **35**, 219.

11 Findlay, G. M., *Brit. J. Exp. Path.,* 1932, **13**, 223.

12 Nicolau, S., *et al., Analele Institului Victor Babes,* 1944, **14**, 266; *Experiments Dealing with Epidemic Hepatitis Virus,* Bucarest, 1944, 34 pp. (Roumanian language).

15088

Effect of Rigid Sodium Restriction on Blood Pressure and Survival of Hypertensive Rats.

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During the course of our studies on experimental hypertension in the rat, we have investigated the effects of various types of diets on the blood pressure. In view of the demonstrated effects of excessive amounts of certain amino-acids (particularly cystine) on the kidney, it was thought possible that these effects might be reflected by changes in the blood pressure. The effect of diets varying in their composition, or those in which the vitamin or amino-acid contents were altered, showed relatively little or no effects on the blood pressure. The addition of sodium chloride even in very excessive amounts to the normal diet also raised the blood pressure only slightly. On the other hand, when diets which were naturally low in sodium content were administered without the addition of the usual salt mixture, prompt reduction in the blood pressure occurred to relatively normal levels. The effect of the restriction of the sodium content of the diet on the blood pressure was so impressive as to warrant the experimental trial of such diets in the treatment of human hypertensive patients as described elsewhere.[1] The present paper is limited to our results on the experimental animal.

Methods. Our studies have utilized the rat for reasons discussed elsewhere.[2] Renal hypertension was induced by the procedure described previously.[3] A stock diet (Rockland Farm rat ration) was powdered and dialyzed to remove the diffusible constituents. The food was powdered, suspended in water and placed in Visking tubing. The filled tubing was then placed in cylinders through which a stream of cold water circulated. After 4 or 5 days, tests for chloride in the suspension were negative. Because this procedure induced the loss of diffusible vitamins and inorganic salts other than sodium, the dried dialyzed diet was modified by the addition of synthetic vitamins (thiamin, riboflavin, hexabione, calcium pantothenate and niacin), cod-liver oil and the salt mixture of Osborne and Mendel[4] from which all sodium salts were excluded.

Results. Fig. 1 shows a typical experiment in which a marked decline in blood pressure is induced in the hypertensive rat by the dialyzed diet described. Similar declines in blood pressure were observed following administration of the following substances, either singly or in mixtures: soy bean meal, peanut flour, cooked potatoes, rice, starch and gelatin. All of these substances are naturally low in sodium content.

Fig. 2 shows a decline in blood pressure brought about in groups of 6 rats fed on diets composed entirely of ground peanuts, soy beans, and potatoes. When sodium chloride in sufficient amount was added to these diets or to the dialyzed stock diet, the declines in blood pressure did not occur. The effect of sodium chloride is also shown in Fig. 3, in which the responses to diets of rice with the addition of varying amounts of sodium chloride are indicated. It appears that even 0.5% sodium chloride partially prevents the hypotensive effect of a low-sodium diet, and that 2% sodium chloride completely inhibits the effect. When potassium chloride was

* Aided by grants from Mr. Joe Werthan and Mr. Alfred Starr.

1 Grollman, A., Harrison, T. R., Mason, M. F., Baxter, J., Crampton, J., and Reichsman, F., *J. Am. Med. Assn.*, in press.

2 Grollman, A., *New York Acad. Sci.*, 1945, in press.

3 Grollman, A., PROC SOC. EXP. BIOL. AND MED., 1944, **57**, 102.

4 Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1919, **37**, 557.

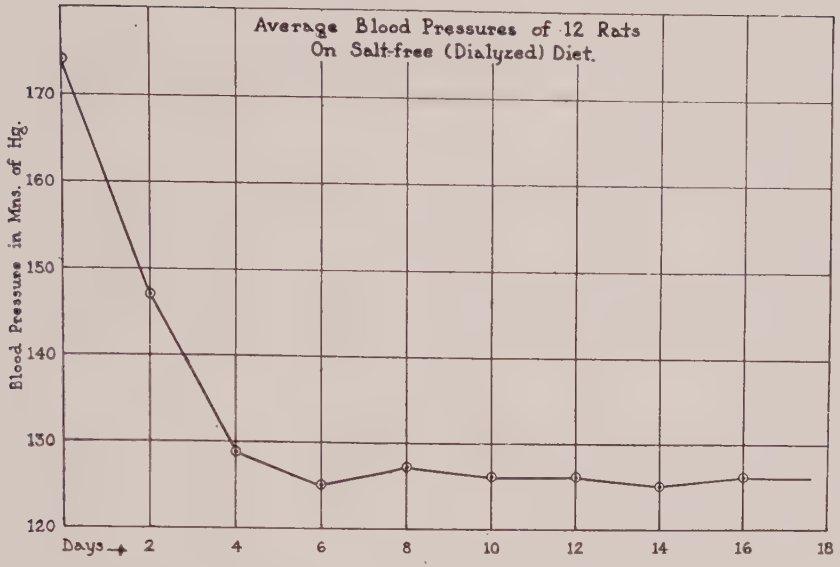

FIG. 1.

The effect of a diet practically free of sodium on the blood pressure of a group of 12 hypertensive rats. The ordinates are averages of the mean pressures on all 12 animals; the abscissæ, days during which the animals were maintained on the diet.

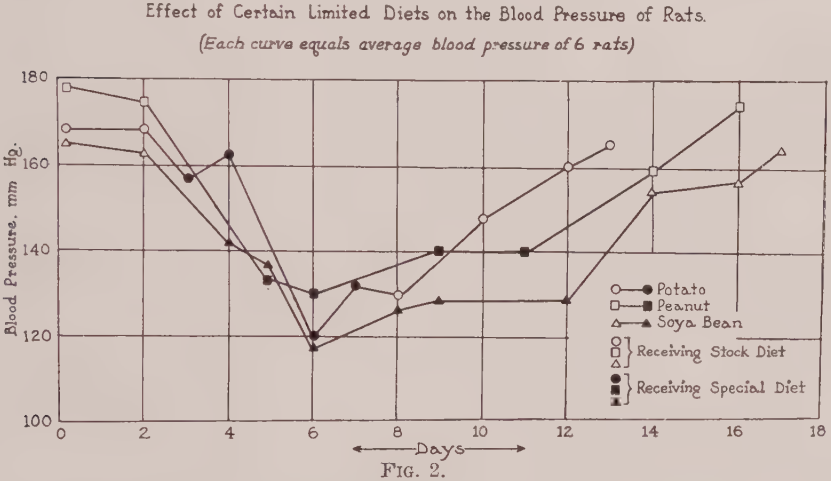

FIG. 2.

The decline in blood pressure which resulted from diets limited to potato, peanut, and soy bean is shown. When the stock diet was resumed the blood pressure rapidly rose toward the pretreatment level.

added to these diets in place of sodium chloride the blood pressures still declined. Hence, as shown in Fig. 4, it appears that deficiency of sodium rather than of chloride is related to the observed hypotensive effect. It should be emphasized that these significant declines in blood pressure were observed in hypertensive animals but not in animals with normal blood pressures in which sodium restriction over short periods of time induces no perceptible drop in blood pressure. Conversely the addition of excessive amounts of sodium chloride to normal diets also as previously shown[5] fails to increase the blood pressure of hypertensive or normal rats.

[5] Grollman, A., Harrison, T. R., and Williams, J. R., Jr., *J. Pharm. and Exp. Therap.*, 1940, **67**, 76.

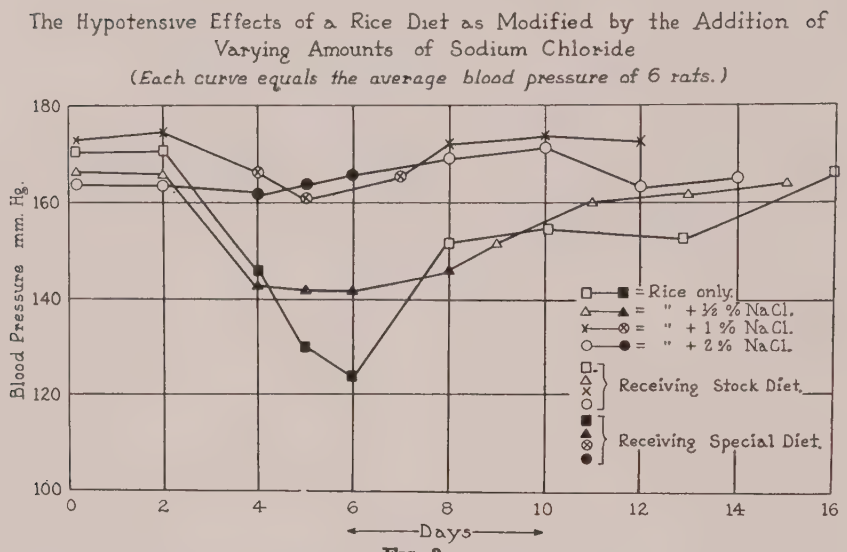

FIG. 3.

When the diet consisted of rice only a well marked decline in blood pressure occurred. The addition of sodium chloride in increasing amounts caused progressive inhibition of the decline in blood pressure.

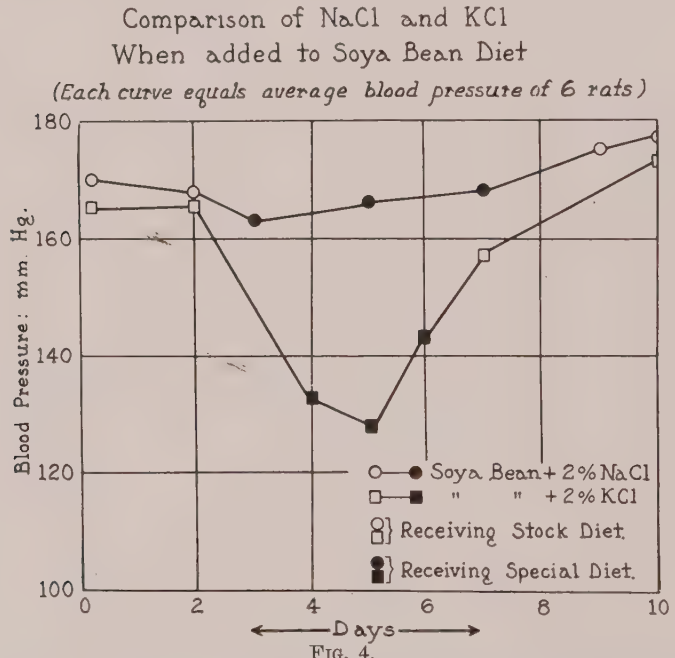

FIG. 4.

The addition of sodium chloride inhibited the hypotensive effect of the soya bean diet. Potassium chloride had no such effect.

Effect on Survival. In order to determine whether or not the marked reduction in blood pressure induced by sodium restriction for long periods is deleterious to previously hypertensive animals, 62 hypertensive rats were divided into two equal groups, matched as to their blood pressures. One group received dialyzed stock diet, supplemented with salts

and vitamins, as described above, and the other received the regular stock diet. At the end of the 100-day period during which this experiment was continued, 15 of the treated animals survived as compared to only 8 of the control group.

The experiment just described seems to indicate that the decline in blood pressure associated with a low-sodium diet is not only not detrimental to animals with experimentally induced hypertension but appears to be beneficial insofar as duration of life is concerned.

Inhibitory Effect of Choline. Among the various vitamins which we have tested for their effect on the blood pressure, choline appeared to exert a slight but definite pressor action in animals with a mild degree of hypertension. When the substance was administered in high dosages (3% of the dried weight of the diet) together with a low sodium diet, the usual drop in blood pressure was not elicited. The mechanism of this inhibitory effect is not clear but unless one assumes that choline in large doses interferes with the excretion of sodium by the kidney of the hypertensive animal, we must consider the effect as possibly being due to the pharmacodynamic action of choline in such large doses.

Discussion. Many studies are available[6] on the effect of the restriction of salt on the blood pressure of human hypertensives but no previous study of this subject has been made on the experimental animal. Although the results on the human have been equivocal,[7] the present study can leave little doubt as to the possibility of reducing the blood pressure of the hypertensive rat by rigid sodium restriction. The mechanism whereby this effect is induced is not established. It is known that there is an accumulation of salt and water in the tissues of the hypertensive patient, which was designated by Ambard[8] as a "dry edema." It would appear most likely, therefore, that when subjected to a drastic sodium restriction the animal with experimental renal hypertension and certain (but by no means all) patients with hypertension rid themselves of the plethora of salt and water in their tissues and that this secondarily results in the observed reduction in blood pressure.

Summary. Drastic sodium restriction was shown to result in a marked drop in the blood pressure of rats with experimental hypertension. This drop in blood pressure was demonstrated to be accompanied by a prolongation of the survival of the animals. The drop was inhibited by the administration of large doses of choline.

6 Allen, F. M., and Sherrill, J. W., *J. Metab. Res.*, 1922, **2**, 429.

7 Fishberg, A., *Hypertension and Nephritis*, Lea and Febiger, Philadelphia.

8 Ambard, L., and Beaujard, E., *La Semaine Med.*, 1905, **25**, 133.

15089

Penicillin Resistant Staphylococci.

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Cultures of staphylococci may readily be made resistant to penicillin *in vitro* as demonstrated by various workers.[1,2] Rammelkamp and Maxon[1] and others[3,4] have also shown that staphylococci may acquire a fastness to penicillin in the human body during the course of therapy with this agent. Further work is necessary before one can safely at-

1 Rammelkamp, C. H., and Maxon, T., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 386.

2 McKee, C. M., and Houck, C. L., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 33.

3 Gallardo, E., *War Medicine*, 1945, **7**, 100.

4 Lyons, C., *J. Am. Med. Assn.*, 1943, **123**, 1007.

tempt to evaluate the significance of acquired *in vivo* resistance to penicillin which bacteria may acquire. Investigation by Rake and collaborators[5] indicate that this phenomenon may not be of great consequence therapeutically due to a loss of virulence by organisms which undergo this change. This is in direct contrast to bacteria which develop sulfonamide fastness; the virulence of such organisms remains unimpaired.

Of much greater importance is the occurrence in nature of virulent staphylococci which are naturally resistant to penicillin. Kirby[6] recently reported the isolation of 7 strains of staphylococci which were naturally resistant to this antibiotic. From each of the 7 strains this worker succeeded in extracting penicillinase,[7,8,9] an enzyme capable of destroying penicillin. From a clinical point of view the occurrence of resistant staphylococci in large numbers could be of great importance. The study to be reported in this paper was undertaken to determine the prevalence and significance of naturally resistant staphylococci.

Materials and Method. Strains of staphylococci studied were isolated from patients in Temple University Hospital. Single colonies were picked from aerobic blood agar plates following incubation at 37° C for 24 hours. No more than one subculture was made from any one specimen or any one patient. Colonies of staphylococci were chosen without regard for hemolysis or pigmentation. The 115 strains studied were from a variety of infections. As indicated by the large number of coagulase-negative reactors many of the strains probably were not of pathognomonic significance and only part of the normal body flora. Subcultures made on infusion agar slants were stored at room temperature and studied in groups of 25 to 30.

The susceptibility of the strains to penicillin was determined by use of a series of beef extract agar plates containing three-fold concentrations of penicillin. Such plates were streaked with 18-hour broth cultures of the staphylococci under investigation. Plates were incubated at 37° C and readings were made at the end of 24 and 48 hours for inhibition of growth.

TABLE I.
Penicillin Resistance and Penicillinase Production of Staphylococci.

Least concentration of penicillin inhibiting growth of staphylococci		Penicillinase production
Units/ml	No. of strains	
0.05	90	—
0.15	9	—
0.45	0	—
1.35	7	+
4.05	7	+
>4.05	2	+

Penicillinase determinations were made on all 115 strains by adding penicillin to broth cultures and subsequently testing for destruction of penicillin by the Oxford cup method as previously reported.[8] Coagulase tests were carried out in the routine fashion. Plasma diluted with 4 parts of 0.85% NaCl in 0.5 ml volumes were inoculated with a loopful of a 24-hour broth culture. These tests were placed in the incubator at 37° C for 4 to 5 hours and left at room temperature over night. Any evidence of a coagulum was accepted as a positive test.

Results. In general the cultures of staphylococci studied fell into 2 distinct groups as to their sensitivity to penicillin. All of the susceptible strains were inhibited by 0.15 units of penicillin per ml of agar. Most of the strains were inhibited by 0.05 units. Resistant strains, on the other hand, required penicillin in concentrations 10 to 25 times greater for the same degree of inhibition. Many of these required as much as 4.0 units before inhibition took place. Table I summarizes the results of these tests. Sixteen strains or 13.9% of the staphylococci studied were resistant to penicillin as evidenced by the necessity for a concentration of penicillin greater than 0.15 units for complete inhibition.

Close correlation is observed between resistance of staphylococci to penicillin and

5 Rake, G., McKee, C. M., Hamre, D. M., and Houck, C. L., *J. Immunol.*, 1944, **48**, 271.

6 Kirby, W. M., *Science*, 1944, **99**, 452.

7 Abraham, E. P., and Chain, E., *Nature*, 1940, **146**, 837.

8 Bondi, A., and Dietz, C. C., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 132.

TABLE II.
Coagulase Activity and Penicillinase Production of Staphylococci.

Coagulase activity	Penicillinase production	
	Positive	Negative
Positive	12	54
Negative	4	45
Totals	16	99

their ability to produce penicillinase. Each strain requiring a concentration greater than 0.15 units for inhibition was found to produce penicillinase. On the other hand not a single susceptible strain produced penicillinase. It is apparent from Table II that the ability to produce coagulase is rather common among the penicillinase-positive staphylococci.

Discussion. It is clinically significant that such a large number of staphylococci are naturally resistant to penicillin. The incidence of 13.9% observed in this study is in general agreement with that of Spink and collaborators,[10] 12.0%, and that of Gallardo,[3] 12.9%. Infections caused by such staphylococci are not likely to respond to penicillin in dosages generally prescribed.[11] It remains to be seen whether increased dosage might be effective against such infections. No doubt certain of the therapeutic failures which have been reported[12] were infections caused by these naturally resistant staphylococci. The routine *in vitro* testing of staphylococci to penicillin prior to treatment would be of definite value in the prediction of such failures.

The perfect correlation which exists between resistance of staphylococci to penicillin and their ability to produce penicillinase is worthy of note. It is apparent that their resistance is the result of their ability to produce penicillinase. This is not true of other penicillin resistant bacteria.[13] Organisms such as *E. typhosa* and *Salmonellas* are resistant to penicillin but they do not produce penicillinase. Members of the genus *Shigella* and other organisms produce this enzyme but whether this is the only factor in their resistance to penicillin remains to be seen. As far as staphylococci are concerned one could run a penicillinase test in order to determine their susceptibility to penicillin. Ability to produce penicillinase may be accepted as *a priori* evidence of resistance to penicillin.

There seems to be no general relationship between coagulase production and penicillinase production. Of the 16 strains producing penicillinase 12 or 75.0% were coagulase positive. Inasmuch as the ability to produce coagulase is generally accepted as an important criterion of the vriulence of staphylococci, it is significant that so large a number of resistant staphylococci are coagulase positive.

There is but little doubt in the opinion of the authors that the resistance of the 16 strains of staphylococci to penicillin is a natural rather than an acquired resistance. This opinion is based upon previous work of the authors[13] as well as that of other workers, namely, that organisms made resistant to penicillin either *in vitro* or *in vivo* do not acquire the ability to produce penicillinase. The mechanism involved in such induced resistance of bacteria to penicillin is as yet unknown, but it is quite definite that the development of such resistance is not accompanied by an acquisition of the ability to produce penicillinase. That all 16 of the staphylococci produced penicillinase appears to the authors to be sufficient evidence for the claim that the resistance is a natural one. In the case of staphylococci the penicillinase test should prove to be an important tool in the study and differentiation between natural and acquired resistance to penicillin.

Summary. (1) In a study of the susceptibility of 115 strains of staphylococci to penicillin 16 or 13.9% were found to be resistant. (2) All strains resistant to penicillin pro-

9 Woodruff, H. B., and Foster, J. W., *J. Bact.*, 1944, **47**, 425.

10 Spink, W. W., Ferris, V., and Vivino, J. J., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 207.

11 Kolmer, J. A., *Penicillin Therapy*, D. Appleton-Century Co., 1945.

12 Bloomfield, A. L., Kirby, W. M., and Armstrong, C. D., *J. Am. Med. Assn.*, 1944, **126**, 685.

13 Bondi, A., and Dietz, C. C., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 135.

duced penicillinase; none of the susceptible strains had this property. (3) Many of the penicillinase-producing staphylococci produce coagulase indicating the importance of the former in infection. (4) Staphylococci naturally resistant to penicillin appear to be resistant as a result of their ability to produce penicillinase.

15090

Relationship Between Precipitative and Protective Antibodies of Type III *Shigella paradysenteriae* (Flexner) Immune Serum.

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The isolation and properties of the specific somatic antigens of several types of *Shigella paradysenteriae* (Flexner) have been described in detail in a previous publication.[1] These substances appear to be single chemical entities constituted from a phospholipid, a protein and a polysaccharide. The antigens are toxic and as far as has been determined, account for all of the immunological properties of the microorganisms from which they are derived. Thus, when small quantities of antigen are injected into experimental animals they give rise to bacterial agglutinins and precipitins which are similar in all respects to those evoked by the microorganisms themselves. In addition, they elicit antibodies in mice which protect them against lethal infections with homologous bacilli. The polysaccharide portion of the complex is neither toxic nor is it antigenic in rabbits; it is this constituent, however, which is responsible for most of the serological properties of the somatic antigen.

The present study was undertaken to determine whether the specific lipocarbohydrate-protein complex of Flexner dysentery bacilli is the only antigen which stimulates the production of protective antibodies or whether the microorganisms contain additional constituents which are important in the immune response. Experiments designed to ascertain the immunological role of the polysaccharide hapten are included as well.

In the following experimental account we shall compare the protective antibody present in the sera of rabbits immunized with Type III Flexner bacilli with that remaining after absorption with the homologous specific antigen and its polysaccharide hapten.

Materials and Methods. The Type III specific antigen used in this study was isolated as described previously.[1] The polysaccharide hapten was obtained by dissociation of the complex with acetic acid.[1] The immune sera were prepared by subjecting rabbits to 3 prolonged courses of injections with formol-killed *Sh. paradysenteriae* (Flexner) Type III (Andrewes and Inman,[3] Type Z, Boyd,[2] and Weil *et al.*,[4] Type III). The rabbits were bled after each course of injections and the sera obtained were pooled. Passive protection tests were performed on Swiss mice weighing 18-20 g. 0.1 ml of 5-fold dilutions of the sera to be tested were injected intra-

* Part of the work described in this paper was done under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and The Rockefeller Institute for Medical Research.

1 Goebel, W. F., Binkley, F., and Perlman, E., *J. Exp. Med.*, 1945, **81**, 315.

2 Boyd, J. S. K., *Trans. Roy. Soc. Trop. Med. and Hyg.*, 1940, **33**, 553.

3 Andrewes, F. W., and Inman, A. C., *Med. Research Council*, Special Report Series, No. 42, London, 1919.

4 Weil, A. J., Black, J., and Farsetta, K., *J. Immunol.*, 1944, **49**, 321.

TABLE I.
Results of Mouse Protection Tests of Type III Immune Serum Before and After Absorption with Specific Antigen and Hapten.

Exp. No.	No. of micro-organisms in challenging dose	Ml of serum required to protect 50% of the mice		
		Serum absorbed with specific antigen	Serum absorbed with hapten	Unabsorbed serum
1	16×10^6	—	—	0.00003
2	4×10^6	—	—	0.000025
3	1.4×10^6	0.003	—	0.00005
4	9×10^6	0.0008	0.00015*	0.000035
5	32×10^6	0.0008	0.00015*	0.000015
6	4×10^6	—	0.00025†	0.000075
7	4×10^6	—	0.0001†	0.00003
Mean		0.0015	0.00016	0.000037 ± 0.000018

50% of untreated control mice were killed by 1 to 9 microörganisms of the virulent Type III Strain No. 79-118-5Z.

* Serum absorbed with slight excess of hapten.

† Serum absorbed with great excess of hapten.

peritoneally into each of 6 to 8 mice together with the challenging dose of microorganisms. The test dose consisted of 0.4 ml of a 10^{-2} dilution of a 6-hour culture of mouse virulent Type III (strain 79-118-5Z†) organisms diluted in a 6.25% suspension of Wilson's granular mucin (Type 1701-W). The details of the method are described in a separate communication.[5] Each experiment was repeated at least once. The results given in the accompanying table are expressed in terms of the 50% survival endpoint.[6]

Experimental. I. Absorption of Type III antiserum with somatic antigen. The pooled Type III antiserum was titrated quantitatively with varying amounts of the purified specific antigen. Each ml of serum was found to contain a total of 0.312 mg of precipitin nitrogen. It was also observed that at the equivalence point 395 μg of antigen were required to precipitate 0.296 mg of antibody nitrogen from each ml of serum. A test of the supernatant liquid after precipitation with this quantity of antigen revealed that it still retained 6% of the original precipitin, but contained only a very slight excess of specific antigen amounting to 2 μg/ml. In order to avoid the toxic effects of excess antigen, each ml of full strength serum was absorbed by the addition of an equal volume of a solution containing 395 μg of somatic antigen per ml. The mixture was incubated at 4° C for 48 hours and then centrifuged in the cold. The supernatant serum was used for the mouse protection tests as described above.

The results of the mouse protection tests performed with the serum before and after absorption with the specific antigen are summarized in Table I. In Table II these results are compared with those of the quantitative studies described above. From the data presented it can be seen that absorption of Type III antiserum with homologous antigen removes approximately 95% of the precipitin and approximately 97% of the protective antibodies. The amount of protective antibody which remains after absorption is roughly equivalent to the remaining precipitin.

II. Absorption of Type III antiserum with the homologous polysaccharide hapten. The pooled Type III antiserum was absorbed with a slight excess of specific Type III hapten. The addition of an equal volume of a solution containing 375 μg/ml of the polysaccharide sufficed to remove all of the antibody precipitable by hapten. After absorption, the supernatant was found to contain an excess of polysaccharide amounting to 84 μg/ml. The amount of antibody removed by

† Kindly supplied by Capt. C. V. Seastone, Army Medical School.

5 Weil, A. J., and Farsetta, K., *J. Immunol.*, in press.

6 Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

TABLE II.
Precipitative and Protective Antibodies Removed from Immune Serum by Absorption with Specific Antigen and Hapten.

Immune serum	Antibodies removed as determined by: Quantitative estimations on immune precipitates %	Quantitative estimations on supernates %	Mouse protection tests %
Absorbed with specific antigen	95	94	97
Absorbed with hapten	—	90	77

the polysaccharide hapten corresponded to 90% of that precipitable by the complete antigenic complex. From the mouse protection tests recorded in Tables I and II it can be seen that absorption of the serum with hapten removed approximately 77% of the protective antibodies.

The pooled antiserum was also absorbed with a large excess of Type III hapten (3000 μg per ml of serum). The results of the mouse protection tests with this serum are recorded in Table I. These results do not differ significantly from those obtained with the serum absorbed with a slight excess of hapten.

From the above experiments it is evident that the immune bodies in dysentery antiserum which confer passive immunity on mice are those primarily directed against the somatic antigen. The fact that some 77% of the protective antibodies can be absorbed with the polysaccharide hapten suggests that the major portion of these immune bodies are directed against the carbohydrate component of the complex antigenic molecule.

Summary. 1. Antisera prepared by prolonged immunization of rabbits with Type III *Shigella paradysenteriae* (Flexner) contain mouse protective antibodies which can be removed by absorption with the chemically purified type specific antigen. Absorption of the serum with the polysaccharide portion of the antigenic complex removes approximately 80-90% of the precipitating and protective antibodies.

2. Most, if not all, of the protective antibodies present in Flexner Type III antiserum are directed against the homologous specific antigen. Any protective antibodies reactive with other constituents of the bacterial cell appear to have little or no significance in the immune response. The polysaccharide hapten is the component of the antigenic complex most important in orienting the protective and precipitative antibodies.‡

‡ Similar results are reported by Smolens, J., Halbert, S. P., Mudd, S., Doak, B. W., and Gonzalez, L. M., *J. Immunol.*, in press.

15091

Subtilin—An Antibiotic Produced by *Bacillus subtilis*. I. Action on Various Organisms.*

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Although it has been known for many years[1,2] that cultures of *Bacillus subtilis* are antagonistic to the growth of other organisms,

* This and subsequent investigations on subtilin are part of a cooperative study undertaken by the Department of Pharmacology and Experimental Therapeutics, University of California Medical School, San Francisco, under the direction of Dr. H. H. Anderson; the Department of Bacteriology, University of California, Los Angeles, under the direction of Dr. A. J. Salle; and the Biochemical

comparatively few studies are recorded in the literature. Several workers[3–7] found that *B. subtilis* produced a lytic action on a number of bacteria. The organism has been shown to be antagonistic to *Mycobacterium tuberculosis*,[8] the virus of vesicular stomatitis,[9] and a number of other pathogenic and saprophytic fungi.[10–12] In general, Gram-negative organisms were not appreciably affected by *B. subtilis*.

The subtilin used in these investigations was a purified product prepared from *B. subtilis* grown in a synthetic medium and

TABLE I.
Organisms Susceptible to the Action of Subtilin.

Alcaligenes viscosus
Bacillus anthracis
" *cereus*
" *megatherium*
Corynebacterium diphtheriæ
Diplococcus pneumoniæ I (parent strain)
" " I (sulfa-resistant)
" " II CH (parent strain)
" " II CH (sulfa-resistant)
Gaffkya tetragena
Lactobacillus casei
" *delbruckii*
" *fermenti*
pentosus
Micrococcus ureæ
Mycobacterium phlei
" *smegmatis*
Neisseria catarrhalis
Rhodococcus roseus
Sarcina lutea
" *ureæ*
Staphylococcus aureus (sulfa-resistant)
" "
" " (hemolytic)
" " (Oxford)
" *citreus*
Streptococcus fæcalis
" *lactis*
" *pyogenes* (hemolytic)

TABLE III.
Organisms Not Susceptible to the Action of Subtilin (1:1000).

Aerobacter aerogenes
Alcaligenes fecalis
Brucella abortus
" *suis*
Eberthella typhosa
Escherichia coli
" " *communior*
Klebsiella pneumoniæ
Pasteurella avicida
Proteus X 19
" *vulgaris*
Pseudomonas aeruginosa
" *fluorescens*
Salmonella paratyphi
" *schottmuelleri*
Serratia marcescens
Shigella alkalescens
" *dysenteriæ*
" *paradysenteriæ*
" *sonnei*
Vibrio comma

TABLE II.
Diameters of Zones of Inhibition of Organisms Susceptible to Subtilin.

Organism	Concentration of subtilin used and diameter of zone of inhibition measured in mm				
	1:1,000	1:10,000	1:100,000	1:1,000,000	1:10,000,000
Alcaligenes viscosus	12	0	0	0	0
Bacillus cereus	17	14	0	0	0
Neisseria catarrhalis	14	12	0	0	0
Rhodococcus roseus	35	29	23	12	0
Sarcina lutea	29	25	20	12	0
Staphylococcus aureus (hemolytic)	17	13	0	0	0
" " (Oxford)	22	18	0	0	0
Streptococcus fæcalis	22	18	10	0	0
" *pyogenes* (hemolytic)	17	12	0	0	0

Division, Western Regional Research Laboratory, Albany, California, under the direction of Dr. Howard D. Lightbody.

The work carried out in the University of California laboratories was supported by a grant from Eli Lilly and Company, Indianapolis, Indiana.

1 Metchnikoff, E., *Ann. Inst. Pasteur*, 1897, **11**, 801.

2 Nicolle, M., *Ann. Inst. Pasteur*, 1907, **21**, 613.

3 Rosenthal, L., *C. R. Soc. Biol.*, 1925, **92**, 78.

4 Rosenthal, L., *C. R. Soc. Biol.*, 1925, **92**, 472.

5 Rosenthal, L., *C. R. Soc. Biol.*, 1925, **93**, 1569.

6 Rosenthal, L., and Duran-Reynals, F., *C. R. Soc. Biol.*, 1926, **94**, 309.

7 Rosenthal, L., and Ilitch, Z., *C. R. Soc. Biol.*, 1926, **95**, 10.

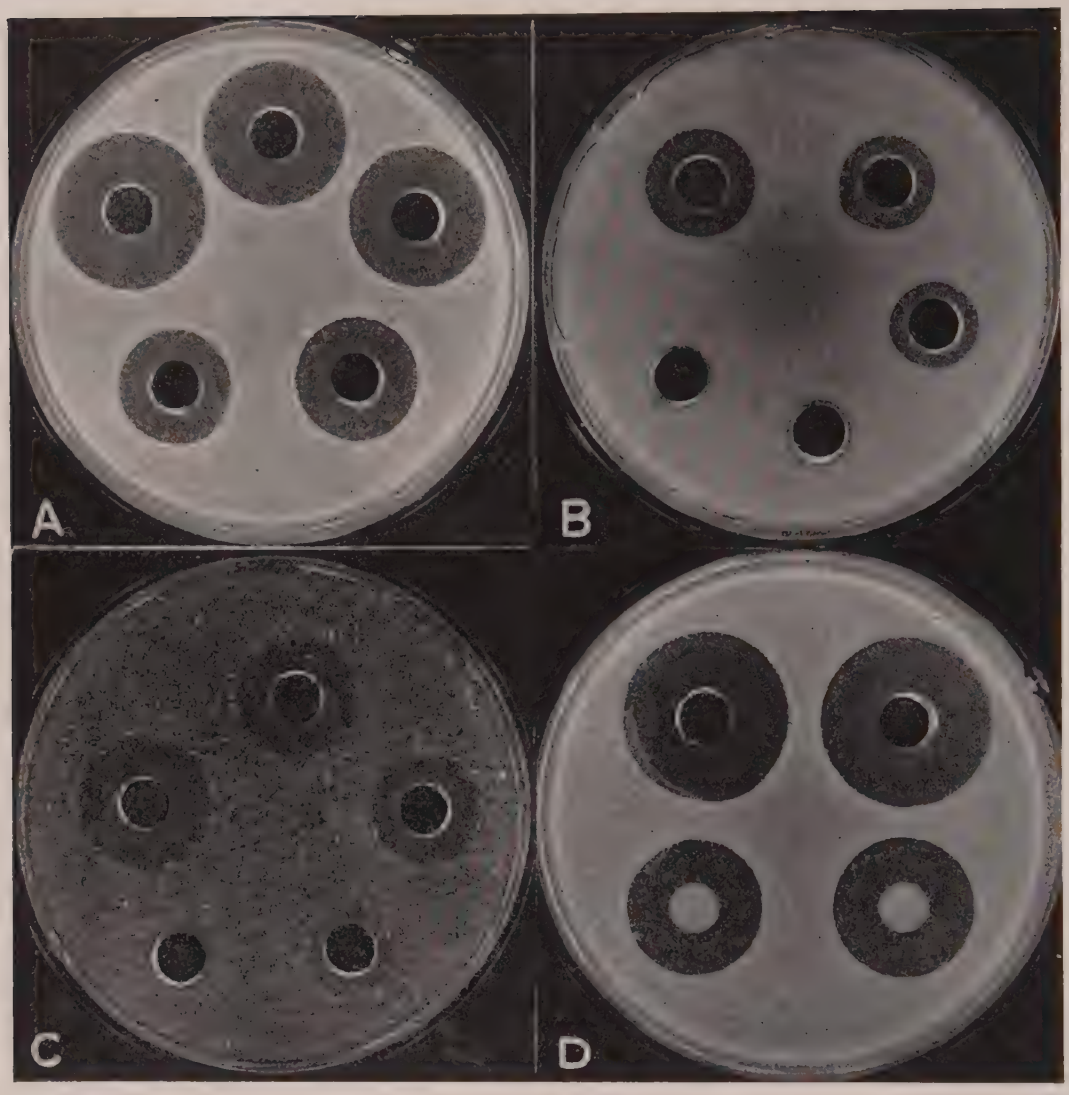


FIG. 1.

Action of subtilin on bacteria by the agar cup-plate and filter paper methods. A. *Sarcina lutea* antagonized by subtilin in concentrations of 1:10,000, 1:50,000, 1:100,000, 1:500,000, and 1:1,000,000. Into each cup was placed 0.1 cc of the subtilin dilution. B. *Staphylococcus aureus.* Dilutions same as in A. C. *Corynebacterium diphtheriæ.* Dilutions same as in A. D. *Sarcina lutea* treated with a 1:10,000 dilution of subtilin. Comparison made between the agar cup-plate and the filter paper methods.

tested for potency against several Gram-positive and Gram-negative organisms.[13] It was prepared by the Biochemical Division of the Western Regional Research Laboratory, Albany, California.

Experimental. Various procedures were

8 Van Canneyt, J., *C. R. Soc. Biol.*, 1926, **95**, 878.

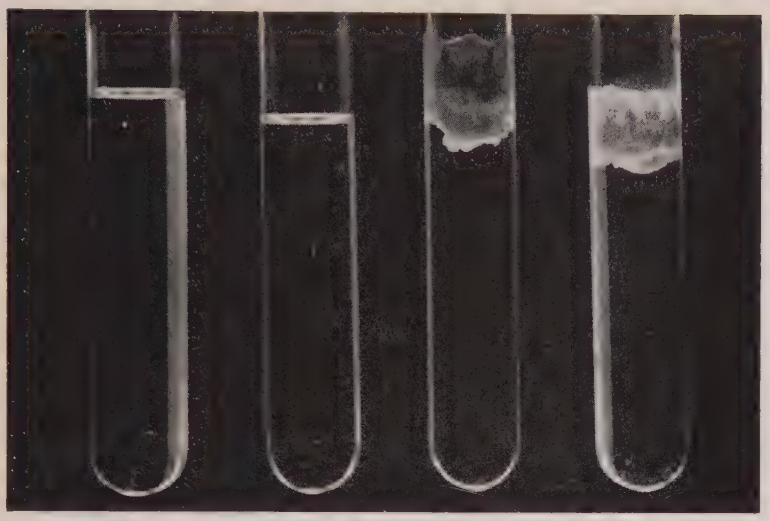
9 Rakieten, M. L., Rakieten, T. L., and Doff, S., *J. Bact.*, 1936, **31**, 55.

10 Bitter, C. Raymond, *J. Colorado-Wyoming Acad. Sci.*, 1941, **3**, 16.

11 Katznelson, H., *Canadian J. Research*, Sect. C, 1942, **20**, 169.

12 Humfeld, H., and Feustel, I. C., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 232.

13 Jansen, E. F., and Hirschmann, Doris J., *Arch. Biochem.*, 1944, **4**, 297.



A B C D

FIG. 2.

Action of subtilin on *Mycobacterium tuberculosis* inoculated into Long's synthetic medium. A. subtilin concentration, 1:33,333; B. subtilin concentration, 1:50,000; C. subtilin concentration, 1:666,666; D, subtilin concentration, 1:100,000.

TABLE IV.
Effect of Subtilin (1:1000) on a Group of Higher Fungi.

Susceptible organisms	Non-susceptible organisms
Actinomyces species (unidentified)	Actinomyces species (unidentified)
Actinomyces asteroides	*Candida albicans*
Actinomyces pelletieri	*Cryptococcus neoformans*
Nocardia mexicana	*Penicillium notatum*
	Sporotrichum schenckii
	Trichophyton gypseum

used to test the activity of subtilin. The method employed depended upon the organism being investigated. To test the action of subtilin on bacteria which grew readily, the following method was used: to 20 cc of melted and cooled agar was added 0.1 cc of a 24-hour nutrient broth culture of the test organism. The inoculated agar was poured into a sterile Petri dish, thoroughly mixed and allowed to harden. The effect of the antibiotic was shown by (1) pipetting 0.1 cc of subtilin dilution into a cup 10 mm in diameter in the agar, or (2) placing on top of the agar a 10 mm disc of filter paper (Whatman No. 2), previously soaked in the subtilin dilution. The inhibitory action of subtilin manifested itself as a clear zone around the agar cup or the disc of filter paper (Fig. 1).

Organisms which were found to be susceptible to the action of subtilin are given in Table I. The concentrations of purified subtilin used ranged from 1:1000 to 1:10,000,000. With the exception of *Neisseria catarrhalis* and *Alcaligenes viscosus,* the organisms are Gram-positive.

Measurements of the diameters of the zones of inhibition of a number of the organisms given in Table I are recorded in Table II.

Organisms not susceptible to the action of subtilin in a concentration of 1:1000 are grouped in Table III. All of the species included in this group are Gram-negative.

Neisseria gonorrhoeae was tested by streaking the organism over the surface of chocolate blood agar, then placing a 10 mm disc of filter paper, previously soaked in a 1:1,000 dilution of subtilin, in the center of the plate. Subtilin was strongly antagonistic to the growth of this organism.

Mycobacterium tuberculosis was inoculated

into tubes of Long's synthetic medium and subtilin added in concentrations ranging from 1:5000 to 1:250,000. The tubes were incubated at 37° C for 3 weeks. The highest dilution of subtilin showing no growth was 1:50,000. At the end of this period of time transfers were made to new medium. The highest dilution showing no growth in the transfer tubes was 1:10,000. These results appear to indicate that subtilin is bacteriostatic in high dilution and germicidal in greater concentration (Fig. 2).

A number of higher fungi were tested by streaking the organisms over the surface of Sabouraud's glucose agar, then placing discs of filter paper soaked in 1:1,000 dilution of subtilin in the centers of the plates. The results are recorded in Table IV.

Summary. The antibacterial product, subtilin, obtained from *Bacillus subtilis* was found to be active chiefly against Gram-positive bacteria. Two notable exceptions to the rule were *Neisseria catarrhalis* and *N. gonorrhoeae,* both Gram-negative, but also antagonized by subtilin. Acid-fast organisms, including *Mycobacterium tuberculosis,* were also found to be susceptible to the antibiotic. The agent produced a bacteriostatic action in high dilution and a germicidal effect in greater concentration. A number of pathogenic higher fungi were also found to be susceptible to subtilin.

15092

Action of Atropine on the Turtle Heart.

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Atropine has at least two significant modes of action on the heart, an early effect due to stimulation of the vagus center, and its well known action as an antagonist of acetylcholine. Concerning an independent direct action on heart muscle, the literature contains conflicting statements. This paper reports a study of the direct action of atropine on the turtle heart *(Pseudemys elegans).* The results indicate that the only significant direct effect of atropine on heart muscle is to depress fiber conduction when the rate of beating is abnormally high. Details of technic have been described in connection with observations on the action of digitalis.[1] The influence on rhythmicity and on contractility was observed when whole auricles or ventricular strips were suspended in a bath and the beat recorded on a smoked drum. Changes in refractory period and conductivity were followed in rhythmically stimulated ventricular strips placed on moistened filter paper; potential differences were amplified, and recorded by a piezoelectric ink writer. Two receiving electrodes were placed on the muscle strip about 15 mm apart, and each was paired with an electrode placed on the paper at a distance of about 2 cm. It has already been shown that the Q-T interval of the electrogram so recorded is an accurate measure of the refractory period, since its duration corresponds closely with measurements made by a direct method.[2] The tissue was observed for an adequate control period before the drug was added.

Effect on Rhythmicity. The effect on a natural pacemaker was studied only in the whole auricle. In the freshly removed auricle, concentrations of atropine varying from 1:200,000 to 1:50,000 caused an increase in rate of beating from 10 to 15% in 4 experiments, while in 3 no change occurred. Con-

[1] Wedd, Blair, and Dwyer, *J. Pharm. Exp. Therap.,* 1941, **72**, 394.

[2] Blair, Wedd, and Young, *Am. J. Physiol.,* 1941, **132**, 157.

TABLE I.
Effect of Atropine on the Q-T and Q-Q Intervals.

Experiment No.	Concentration × 1000	Q-T (sec.) Before	Q-T (sec.) After	Q-Q (sec.) Before	Q-Q (sec.) After
		Rate 13.4 p.m.			
1	1:100	1.86	1.88	0.2	0.2
2	1:100	1.60	1.60	X	X
	1:50	1.60	1.52	X	X
3	1:100	1.40	1.36	X	X
4	1:50	1.36	1.36	0.18	0.18
	1:25	1.36	1.12	0.18	0.18
5	1:33	1.44	1.40	X	X
6	1:20	1.48	1.42	X	X
7	1:25	1.96	1.64	0.18	0.20
8	1:25	1.44	1.48	0.42	0.58
9	1:25	1.60	1.36	.16-.20	.20-.24
10	1:25	1.64	1.60	.24-.36	.36-.40
		Rate 25 p.m.			
11	1:25	0.40	0.40	0.20	0.24
12	1:50	1.24	1.24	0.38	0.52
	1:25	1.24	1.20	0.52	0.64
13	1:25	1.16	1.16	0.28	0.40
14	1:25	X	X	0.26	0.32
15	1:25	X	X	0.36	0.50
16	1:25	1.04	1.08	0.36	0.48

centrations of 1:25,000 caused slight slowing in 3 preparations and no change in one. Observations made on auricles that had been kept cold for 24 or 48 hours gave results similar to those obtained with fresh tissues. Tonus waves when present were unaffected by the drug. Extrasystolic arrhythmias were not seen in auricular preparations and but twice in ventricular; one first appeared and the other disappeared following atropine.

Effect on Contractility. Changes in mechanical response due to atropine were studied in rhythmically driven ventricular strips which were 24 to 48 hours old. The concentrations used varied from 1:100,000 to 1:25,000. Of 8 observations, the strength of beat remained unchanged in 6; in one there was a slight increase, and in one a slight decrease. Tonus, measured by diastolic length, usually showed no change, and when a slight change did occur, the direction was not constant.

Effect on Refractory Period. This was observed by following the Q-T interval in the electrogram of rhythmically driven ventricular strips, which in most instances had been removed 24 hours before the observations were made (Table I). In the first group studied the rate of stimulation was 13.4 beats per minute. In general, concentrations of atropine varying from 1:100,000 to 1:20,000 had little effect; such action as did occur always shortened the Q-T interval; in 3 of 12 experiments the shortening amounted to 15 to 18%. In a second series for which the driving rate was 25 per minute, concentrations of 1:25,000 produced even less change; in 2 there was no change, and in 2 very slight

lengthening of questionable significance. This result was to be expected for the Q-T intervals of the second group were already shortened by the relatively high rate of beating.

Effect on Conductivity. Conduction was studied in those preparations for which excitability changes have just been described. The Q-Q interval is the conduction time for the length of muscle between the receiving electrodes. The values before and after atropine are given in Table I. In most experiments at the slow rate of beating conduction changes were slight or absent. In one in which the interval was considerably lengthened by atropine the initial conduction time was unusually prolonged. However, at the more rapid rate of beating conduction time was always increased. These results are in accord with those of Lewis and Drury[3] who stated that in the dog's auricle atropine had no effect on transmission intervals at rates of 250 per minute or less; they did not doubt that it might increase the intervals at higher rates of beating. Likewise, Megibow and Katz[4] found that in dogs in which auricular fibrillation was maintained by faradic stimulation, A-V conduction was depressed in the denervated animals by a direct action on the conducting tissues. Several experiments of the second group give striking examples of the independence of refractory period and conductivity in heart muscle; the former at high rates of beating tends to become fixed at a shortened interval, while conduction is the more easily slowed.

Summary. The direct action of atropine on cardiac muscle has been studied in the whole auricle and ventricular strips of turtle heart. In concentrations far above those that could be used therapeutically, its effect on rhythmicity, contractility, absolute refractory period, tonus, and on conductivity at low rates of beating was slight and inconstant. The only significant direct action of atropine on heart muscle appears to be a power to depress conductivity at relatively high rates of beating.

[3] Lewis and Drury, *Heart,* 1921, **8**, 83.

[4] Megibow and Katz, *J. Pharm. Exp. Therap.,* 1940, **70**, 388.

15093

Response to Tissue Injury of Lymphoid Tissue Previously Altered by Castration, Thyroidectomy and Thyroid Feeding.

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It has been known for some time that introduction into the body of various damaging agents causes prompt shrinkage of lymphoid tissue.[1] Lymphocytes produce, or at least contain antibodies.[2–4] These two facts have been thrown into a new light by recent work which indicates that injections of adrenal cortical extract, or secretion by the adrenal cortex induced by injections of pituitary adrenotrophic hormone, result in prompt dissolution of lymphocytes,[5,6] which thus release protein and antibodies into the lymph[7,10] with concomitant shrinkage of

[1] Selye, H., *Endocrinology,* 1937, **21**, 169.

[2] Ehrich, W. E., and Harris, T. N., *J. Exp. Med.,* 1942, **76**, 335.

[3] Dougherty, T. F., Chase, J. H., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 295.

[4] Harris, T. N., Grimm, E., Mertens, E., and Ehrich, W. E., *J. Exp. Med.,* 1945, **81**, 73.

[5] Dougherty, T. F., and White, A., *Endocrinol.,* 1944, **35**, 1.

[6] Reinhardt, W. O., and Li, C. H., *Science,* 1945, **101**, 360.

[7] White, A., and Dougherty, T. F., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 26.

TABLE I.
Shrinkage of Lymphoid Tissue After Subcutaneous Injections of Formalin.

	Intact 6 pairs	Gonadectomized 4 pairs	Thyroid fed 2 pairs	Gonadectomized and thyroid fed 2 pairs	Thyroidectomized 3 pairs
Thymus, mean g					
Uninjected rats	.422	.533	.344	.415	.343
Injected "	.267	.367	.277	.303	.143
Difference	—.155	—.157	—.067	—.112	—.200
Lumbar lymph nodes, mean g					
Uninj. rats	.042	.069	.031	.035	.026
Inj. "	.021	.038	.022	.034	.012
Diff.	—.021	—.031	—.009	—.001	—.014
Mesenteric lymph nodes, mean g					
Uninj. rats	.282	.310	.218	.160	.261
Inj. "	.197	.245	.206	.134	.126
Diff.	—.085	—.065	—.012	—.026	—.135
Spleen, mean g					
Uninj. rats	.432	.461	.608	.700	.284
Inj. "	.261	.349	.526	.363	.159
Diff.	—.171	—.112	—.082	—.337	—.125
Adrenals, mean g					
Uninj. rats	.039	.042	.039	.041	.027
Inj. "	.049	.052	.039	.051	.035
Diff.	+.010	+.010	0	+.010	+.008
Body weight, mean g					
Uninj. rats	243	204	264	224	162
Inj. "	243	232	247	253	160
Diff.	0	+ 82	— 17	+ 29	— 2

lymphoid tissue.[11–14] Apparently, injection of damaging agents initiates this chain of events. Under these circumstances the adrenal cortex loses its lipoids as it discharges its secretion, evidence for which has been reviewed.[15] The adrenal cortex then enlarges in response to functional demand for its hormone, unless the damaging agents are of such magnitude as to cause adrenal exhaustion. Indications are that the adrenal factor involved is that promoting gluconeogenesis.[5,16]

As this important protective phenomenon of release of substances from lymphocytes probably depends upon secretion of the pituitary adrenocorticotrophic hormone, it seemed of interest to determine whether experimental conditions, such as castration, thyroidectomy and thyroid feeding, which are known to alter the size of lymphoid tissue[17,18] (and others) and to cause qualitative and quantitative changes in the cellular pattern of the pituitary and in its secretion of hormones, would interfere with the response of lymphoid tissue to toxic substances.

8 Dougherty, T. F., White, A., and Chase, J. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 28.

9 Dougherty, T. F., Chase, J. H., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 135.

10 White, A., and Dougherty, T. F., *Endocrinol.*, 1945, **36**, 207.

11 Evans, H. M., Moon, H. D., Simpson, M. E., and Lyons, W. R., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 419.

12 Ingle, D. J., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 443.

13 Dougherty, T. F., and White, A., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 132.

14 Simpson, M. E., Li, C. H., Reinhardt, W. O., and Evans, H. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 135.

15 Sayers, G., Sayers, M. A., Fry, E. G., White, A., and Long, C. N. H., *Yale J. Biol. and Med.*, 1944, **16**, 361.

16 Venning, E. H., Hoffman, M. M., and Browne, J. S. L., *J. B. C.*, 1943, **148**, 455.

17 Chiodi, H., *Endocrinol.*, 1940, **26**, 107.

18 Reinhardt, W. O., and Wainman, P., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 257.

Groups of litter-mate rats of the same sex were used. Some were (1) castrated, (2) some thyroidectomized, (3) some fed thyroid extract and some (4) gonadectomized and fed thyroid extract. Periods of 29 to 68 days were allowed to elapse after operation so as to allow time for structural changes to result in pituitary and lymphoid tissue. Those fed thyroid extract were given daily .1 g per 100 g body weight for several weeks. Of each pair, one rat was an uninjected control. In the second rat of each pair tissue damage was produced by subcutaneous injections in the legs of .5 cc 4% formalin, twice daily for 2 days, killing the rats on the 3rd day, according to the method used by Selye[11] in intact rats.

The table gives the results. Comparisons should be made between the injected and uninjected rats, in each category, which are litter-mates. As rats of one group, such as the castrated, are not the same age as another group, such as the thyroidectomized, no conclusions have been drawn as to whether rats in one group responded with greater or less degree than those in other groups. However, since the body weights are nearly the same in the different groups, except in the dwarfed thyroidectomized rats, a rough comparison can be made. The results within each group show that the rats injected with formalin had definite shrinkage of lymphoid tissue, no matter what the previous alteration was in lymphoid tissue. In fact, 2 castrated rats which showed chronic infection at the site of operation were not excluded from the series because this additional effect on lymphoid tissue did not prevent its response after acute injury. Since observations made in each group were of the same pattern of response, with few inconsistencies, it was not thought necessary to carry out a larger series of determinations.

Conclusions. Lymphoid tissue (lymph nodes, thymus and spleen) which had been altered by various experimental conditions was capable of shrinkage in response to injury of subcutaneous tissues by formalin injections.

15094

Studies on the Absorption and Excretion of Streptomycin in Animals.

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The introduction of streptomycin as a chemotherapeutic agent[1,2,3] has made apparent the need for a quantitative study of the distribution of this substance in body fluids. The present study was therefore undertaken to determine the relative concentrations of streptomycin in the blood, urine, feces, and tissues of various animal species following oral and parenteral administration.

Materials and Methods. The streptomycin* used in these studies ranged in potency from 150-400 units per mg of solid. The amount of drug administered was based on the findings in the efficacy and toxicity experiments reported by Robinson, Smith, and Graessle.[3]

The animal species used were normal monkeys, dogs, rabbits, and mice. All animals were maintained on adequate stock diet and housed under conditions of controlled temperature and humidity.

In all experiments streptomycin was admin-

1 Schatz, A., Bugie, E., and Waksman, S. A., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 66.

2 Jones, D., Metzger, H. J., Schatz, A., and Waksman, S. A., *Science*, 1944, **100**, 103.

3 Robinson, H. J., Smith, D. G., and Graessle, O. E., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 226.

* This material was prepared by Drs. Robert Denkewalter and Max Tishler from broth supplied by Dr. J. W. Foster of the Research Laboratories of Merck & Co., Inc.

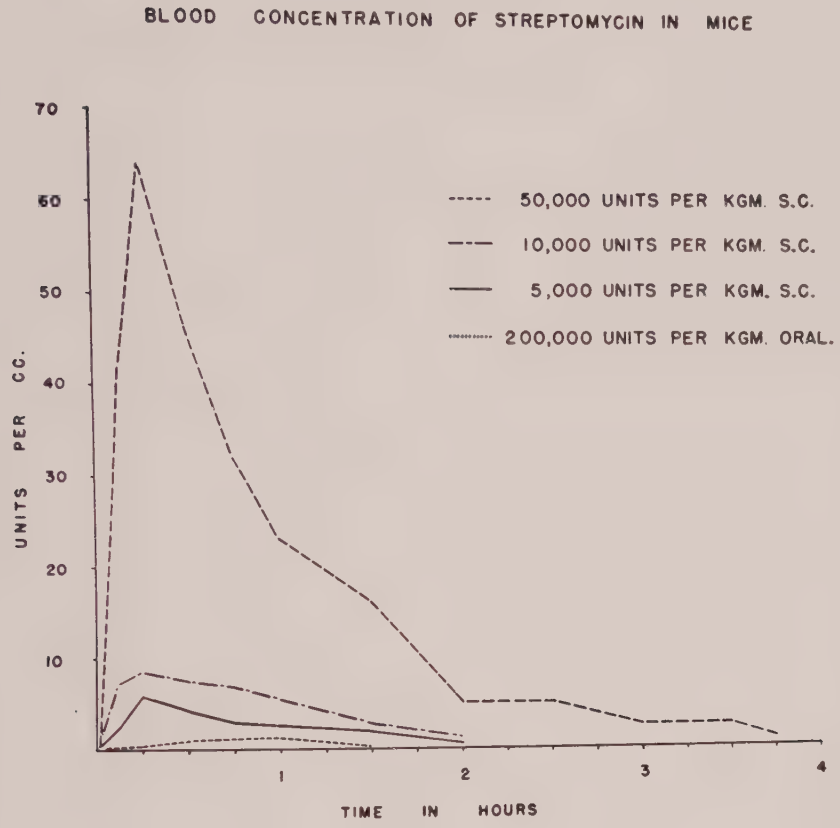

FIG. 1.

istered as an aqueous solution. In order to insure adequate urine flow tap water was administered by stomach tube to each animal 10-15 minutes before giving streptomycin.

Blood and urine samples were taken at frequent intervals. All assays were completed within a 24-hour period. The method of assay, employing *Staphylococcus aureus SM* as the test organism, was that reported by Stebbins and Robinson.[4]

The excretion of streptomycin into the bile was studied in dogs and rabbits. The animals were subjected to deep anesthesia with nembutal, the abdomen opened and the common bile duct cannulated. Samples of bile were obtained at frequent intervals, usually every 15 minutes, and assayed in the same manner as blood and urine.

In those cases where streptomycin was administered orally, or by duodenal instillation, the stools of the gastro-intestinal tract were collected in distilled water, centrifuged, and the supernatant fluid assayed as described above for blood.

Results. Blood Levels. Mice: From the results presented in Fig. 1, it is apparent that streptomycin is rapidly absorbed and excreted following subcutaneous administration in mice. A single therapeutic dose of 5,000 units per kg produced a maximum blood concentration of 6.5 units per ml within 15 minutes, which gradually declined to 2 units per ml during a 2-hour period. Larger doses of 10,000 and 50,000 units per kg subcutaneously resulted in correspondingly higher and more prolonged blood concentrations, although, here again, the drug was rapidly excreted (Fig. 1). Following oral administration of large doses of streptomycin (200,000 units per kg) only small amounts of the drug were de-

[4] Stebbins, R. B., and Robinson, H. J., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 255.

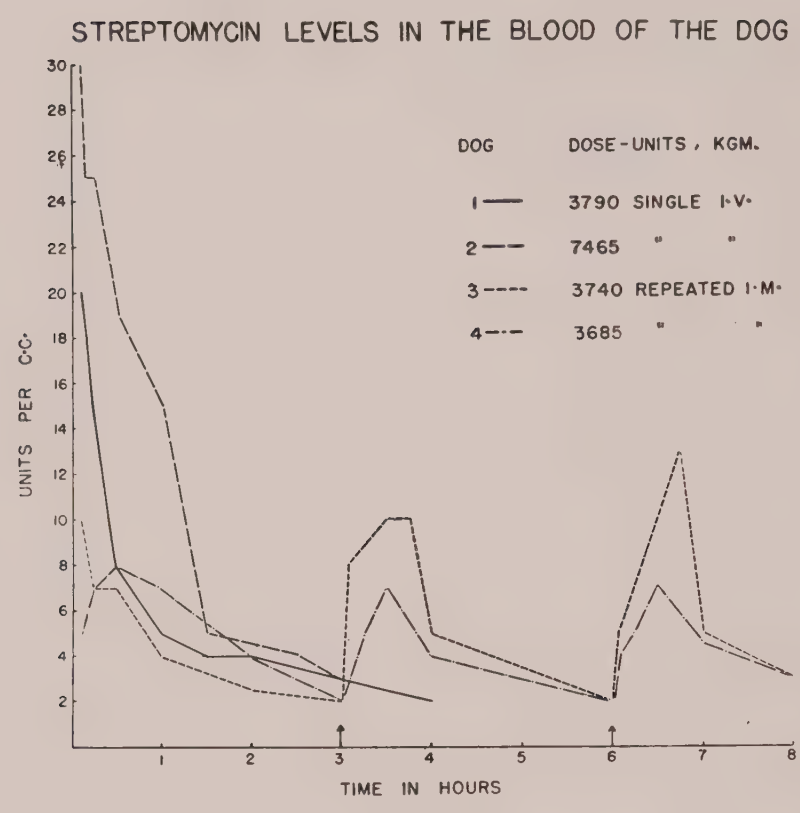

FIG. 2.

tected in the blood (2 units per ml). The blood level curve which was obtained following oral administration of the drug differed from that which was obtained following parenteral injection, in that the maximal concentration following oral administration did not occur until 45 to 60 minutes following drug administration.

Dogs and Monkeys: The findings in dogs and monkeys (Fig. 2 and 3) are similar to those obtained following parenteral administration of streptomycin in mice. Single intravenous dosage of 3700 and 7400 units per kg (100,000 units per dog) of streptomycin produced blood levels of 20 and 30 units per ml respectively, immediately following injection. At the end of 4 hours the blood concentrations decreased to 2-3 units per ml. By repeated intramuscular injection of streptomycin every 3 hours in dogs and monkeys (Fig. 2 and 3) therapeutically effective blood concentrations ranging between 3-18 units per cc could be maintained. Peak blood levels of the drug were reached within 30 minutes after each injection and then gradually declined in much the same manner as that following a single dose.

Urine Levels. The rapid disappearance of streptomycin from the blood following parenteral administration can be largely accounted for by its early appearance in the urine. In a series of ten dogs approximately 50-80% of the drug was excreted in the urine within a 24-hour period. During the first 2 hours after injection 50% of the initial dosage appeared in the urine.

In a group of 2 monkeys receiving 10,000 units per kg of streptomycin per day for 5 days, approximately 60% of the drug was accounted for in the urine during this period (Table I). Examination of the urine collected during the subsequent 5-day period showed that it contained an additional 5-6% although the concentration in the blood was below the level that could be measured.

When the dose of streptomycin was

TABLE I.
Total Urinary Excretion by Monkeys Following Divided Intramuscular Dosage of Streptomycin.

Monkey No.	Time, days	Dose, units/kg × 1000	Total daily dose, units × 1000	Units excreted in 24 hrs × 1000	% daily dose excreted	Total % recovered
1	1	10	28.9	19.9	69	
	2	10	28.9	20.3	70	
	3	10	28.9	14.9	52	
	4	10	28.9	13.6	47	
	5	10	28.9	18.5	63	60
	6	—	—	4.		
	7	—	—	.4		
	8	—	—	1.		
	9	—	—	.6		
	10	—	—	.7		
	11	—	—	.0		65
2	1	10	32.4	19.8	61	
	2	10	32.4	20.5	63	
	3	10	32.4	17.1	53	
	4	10	32.4	16.4	51	
	5	10	32.4	24.5	76	60
	6	—	—	4.3		
	7	—	—	.8		
	8	—	—	1.1		
	9	—	—	1.2		
	10	—	—	.9		
	11	—	—	.0		66
3	1	50	167	70.	42	
	2	50	167	90.	54	
	3	50	167	78.4	47	
	4	50	167	72.	43	
	5	50	167	54.	32	44
	6	—	—	7.2		
	7	—	—	1.6		
	8	—	—	1.3		
	9	—	—	1.1		
	10	—	—	.6		
	11	—	—	.0		45
4	1	50	154	58.4	38	
	2	50	154	37.5	24	
	3	50	154	98.8	64	
	4	50	154	64.	42	
	5	50	154	32.5	21	38
	6	—	—	7.		
	7	—	—	1.4		
	8	—	—	.8		
	9	—	—	.6		
	10	—	—	.3		
	11	—	—	.0		39

increased to 50,000 units per kg in a second group of two monkeys (Table I) and administered by the same dosage schedule, 38-44% of the drug was excreted in the urine during treatment, followed by a further urinary output of 1% of streptomycin in the 5-day period after injection had been discontinued.

Biliary and Fecal Excretion Studies. Assay of the bile obtained from rabbits for an 8-hour period following single intravenous administration of streptomycin (5,000 units per kg) accounted for only 5-10% of the dose.

Dogs receiving 100,000 units per kg by mouth and rabbits which received 8,000 units per kg intraduodenally in a single dose showed no detectable levels in the bile for a similar time period.

Dogs given 100,000-200,000 units per kg perorally on an empty stomach and sacrificed 24 hours later showed 60-80% of the drug

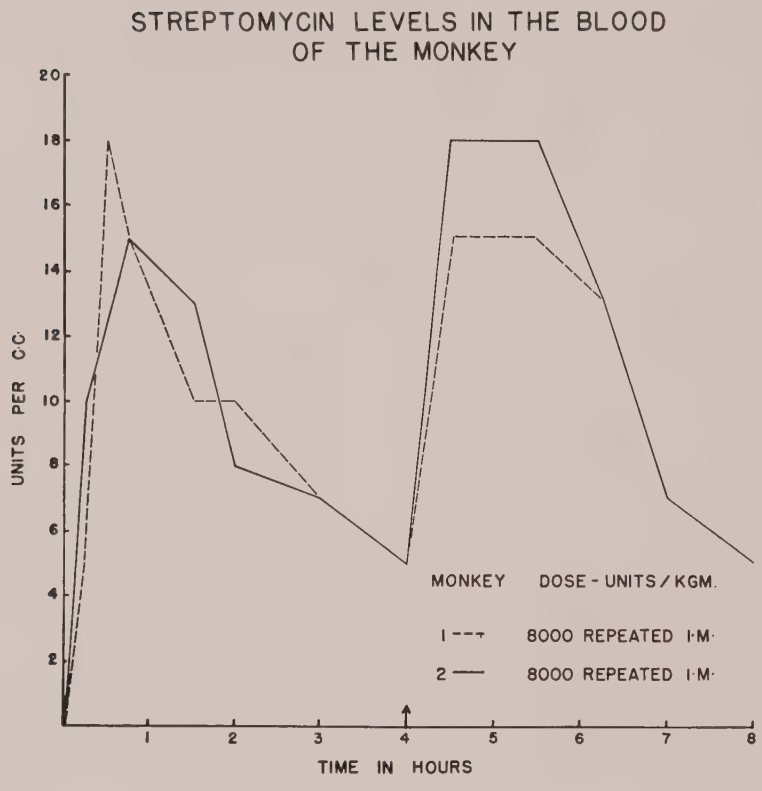

FIG. 3.

unabsorbed from the gastro-intestinal tract. About 5-10% of the drug appeared in the urine during this time. Only traces of streptomycin could be detected in the blood of these animals.

A series of *in vitro* studies on streptomycin in the presence of artificial gastric and duodenal juice indicated that the potency of this antibiotic agent was not materially affected when mixed with these substances for 24 hours at 37.5°C.

Summary and Conclusions. The findings reported in this communication show that streptomycin is rapidly absorbed and excreted following parenteral administration. The rapid disappearance of streptomycin from the blood is largely accounted for by its early appearance in the urine. Approximately 60-80% of the drug is excreted in the urine of dogs within a 24-hour period after parenteral administration. Somewhat smaller amounts were excreted in the urine of monkeys.

When the drug is given perorally relatively small amounts are detected in the blood. This is largely due to the lack of absorption of streptomycin from the gastro-intestinal tract as shown by the large amount of the drug recovered in the feces.

Therapeutic blood concentrations can be maintained by repeated intramuscular injection.

Following intravenous administration of streptomycin, only 5-10% of the dose can be demonstrated in the bile.

15095

Double Lumen Catheter for Intravenous and Intracardiac Blood Sampling and Pressure Recording.*

A. Cournand, R. A. Bloomfield, and H. D. Lauson. (Introduced by W. W. Palmer.)

From the Department of Medicine, College of Physicians and Surgeons, Columbia University; the Department of Physiology, New York University College of Medicine; and the Chest Service, Bellevue Hospital, New York.

As a development of the technic of large vein and intracardiac catheterization,[1,2,3] a double lumen catheter has been devised for the purpose of sampling blood and for recording pressure pulses simultaneously in two adjacent parts of the circulatory system, accessible by means of this technic. These include the internal jugular vein, superior and inferior vena cava, hepatic[4] and renal veins,[5] the right auricle, right ventricle, and pulmonary artery.

The No. 10 catheter retains all the characteristics of the single lumen catheter recently improved,[3] namely, its length, flexibility, X-ray opacity, and curved tip. It is divided internally into two channels (Fig. 1). They are separated by a rigid partition, which as preliminary tests have shown, prevents transmission from one channel to the other of pressure impacts of a magnitude much greater than observed physiologically.

The terminal opening of one lumen is at the catheter tip: the other opens 10 cm back of the tip. At the base or external end of the catheter, each channel ends in a separate tube, with adapter for connecting to syringe, pressure recording apparatus, etc.

Among some of the problems that this new catheter is helping investigate, may be mentioned the following:

1. The persistence of laminar flow in large veins and right auricle; the validity of blood samples from the right auricle as representative of truly mixed venous blood; the general location in the right atrium of the area within which such samples may be more readily obtainable. In Table I are shown all data thus far obtained concerning the CO_2 content and O_2 content in blood samples taken simultaneously from the right auricle and right ventricle.

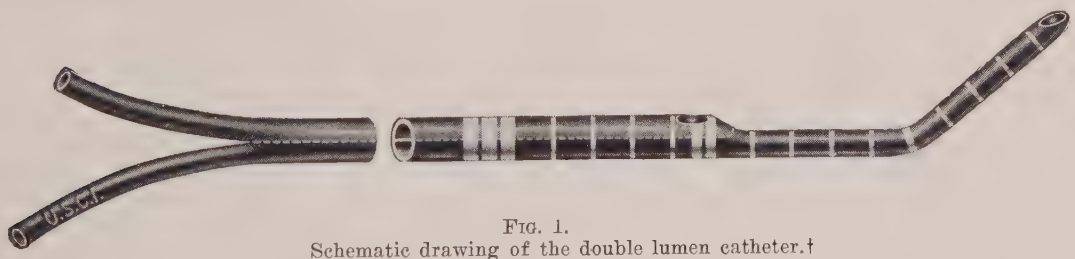

Fig. 1.
Schematic drawing of the double lumen catheter.†

* This investigation was carried on under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University, with the collaboration of New York University. Additional support was provided by the Commonwealth Fund.

1 Cournand, A., and Ranges, H. A., Proc. Soc. Exp. Biol. and Med., 1941, **46**, 462.

2 Cournand, A., Lauson, H. D., Bloomfield, R. A., Breed, E. S., and Baldwin, E. deF., Proc. Soc. Exp. Biol. and Med., 1941, **55**, 34.

3 Cournand, A., Riley, R. L., Breed, E. S., Baldwin, E. deF., and Richards, D. W., Jr., *J. Clin. Invest.*, 1945, **24**, 106.

4 Bradley, S. E., Ingelfinger, F. Y., Bradley, G. P., and Curry, F., to be published.

5 Warren, F. V., Brannon, E. S., Merrill, A. Y., *Science*, 1944, **100**, 108.

† This catheter has been specially designed by the United States Catheter and Instrument Corporation, Glens Falls, New York.

TABLE I.
CO_2 and O_2 Content in Samples Taken Simultaneously from the Right Auricle and Right Ventricle Using a Double Lumen Catheter.

Subject	Date	O_2 content in vol. %		CO_2 content in vol. %	
		right auricle	right ventricle	right auricle	right ventricle
J.K.	6/30/44	9.1	9.3	49.1	49.5
X.B.	7/11/44	17.3	17.1	48.4	48.5
G.B.	7/17/44	8.6	8.8	52.7	52.9
P.W.	10/10/44	14.8	14.8	49.1	49.6
F.McK.	11/14/44	10.1	10.1	43.4	43.9
F.C.	12/19/44	14.4	13.9	49.7	49.7
M.D.	2/20/45	16.2	16.2	50.5	51.7
J.C.	3/20/45	16.5	16.5	48.5	48.4
”	3/20/45	16.4	16.6	47.5	47.4

2. The characteristics of simultaneous mechanical events in the right ventricle and auricle or in the right ventricle and pulmonary artery, in normal individuals, in various types of cardiac failures, or in disturbances of the cardiac rhythm. A few illustrative examples are given in Fig. 2 and 3.

3. The nature of some oscillatory waves appearing on tracings taken from the right ventricle.

4. The demonstration that the presence of the catheter in the right ventricle does not cause tricuspid insufficiency.

The technic of catheterization is not greatly complicated by the larger size of the catheter. However, it can only be used in individuals with fairly large median basilic veins, since it may cause painful veno-constriction when moved along smaller veins. Greater care must be exercised with this type of catheter than with the single lumen, to avoid obstruction by fibrin formation in one or the other of the tubes.

This device has now been used over a period of one year in approximately 20 subjects. No unfavorable side effects other than the local pain mentioned above, from veno-spasm, have been encountered.

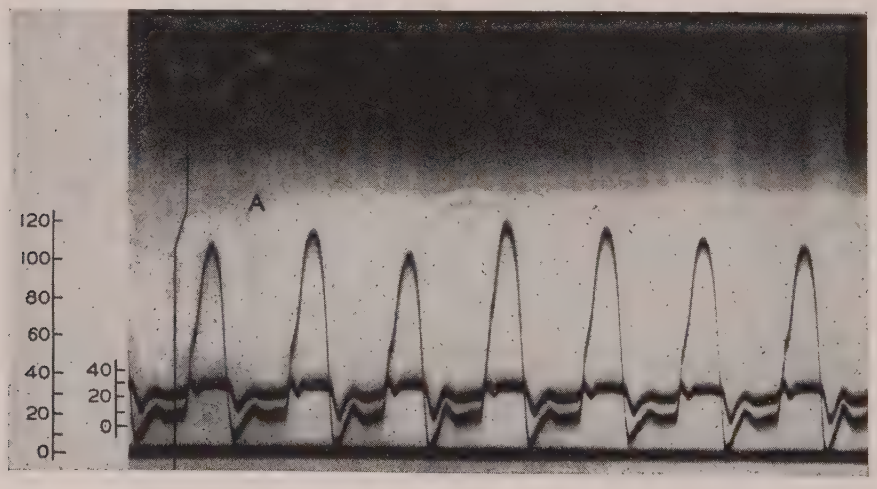

FIG. 2.
Simultaneous recordings in the right auricle and ventricle in a case of rheumatic valvulitis with cardiac decompensation.
Scales to the left in mm Hg. Note (a) the sudden and sustained elevation of pressure on the right auricular tracing during systole, indicating tricuspid regurgitation; (b) the sudden fall of pressure in the right auriele marking the opening of the tricuspid valve; (c) the time and pressure relationship between auricular and ventricular tracings during diastole.

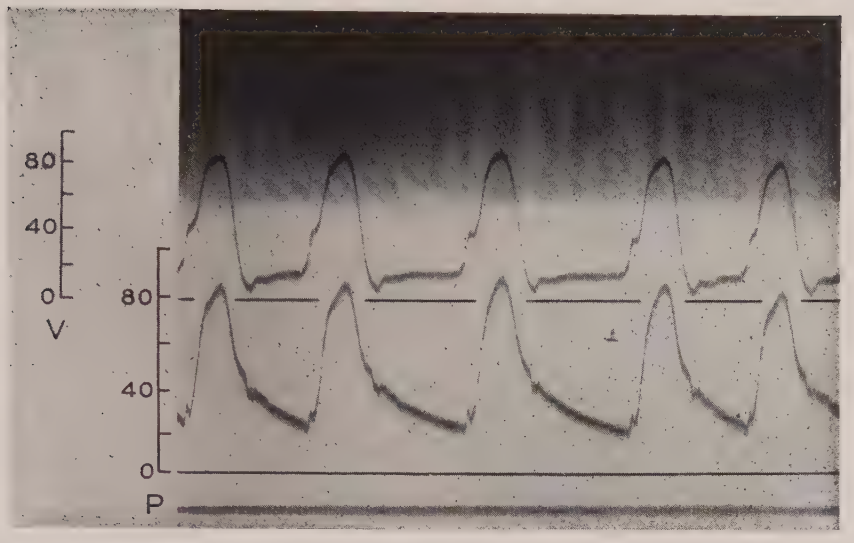

FIG. 3.

Simultaneous tracings in the right ventricle (upper) and in the pulmonary artery (lower) in a case of rheumatic valvulitis with hypertension in the lesser circulation. Note (a) the identity of form and pressure of both tracings during the later part of systole; (b) the small simultaneous oscillations on both tracings during early systole and diastole, presumably due to motion of the catheter in the heart.

Summary. 1. Simultaneous blood samplings and pressure recordings in 2 continuous segments of the circulatory system may be obtained in man by means of a specially designed double-lumen catheter. 2. The difference in oxygen content of 2 blood samples withdrawn simultaneously from 2 points located 10 cm apart in the right auricle and right ventricle was well within the limits of technical error in 8 out of 9 cases. 3. Examples are given of the recording of simultaneous mechanical events in (a) the right auricle and ventricle, (b) the right ventricle and the pulmonary artery.

15096

Simultaneous Registration of Intrathoracic, Right Intracardiac and Systemic Pressure in Man.*

RICHARD A. BLOOMFIELD.† (Introduced by H. W. Smith.)

From the Department of Physiology, New York University College of Medicine, New York City.

Study of the right intracardiac pressures in human subjects, by a technic previously described,[1] has yielded data, for the full interpretation of which a simultaneous record of

* This investigation was carried on under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University, with the collaboration of New York University.

† Present address: Heart Station, Boston City Hospital, Boston, Mass.

1 Cournand, A., Lauson, H. D., Bloomfield, R. A., Breed, E. S., and Baldwin, E. deF., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 34.

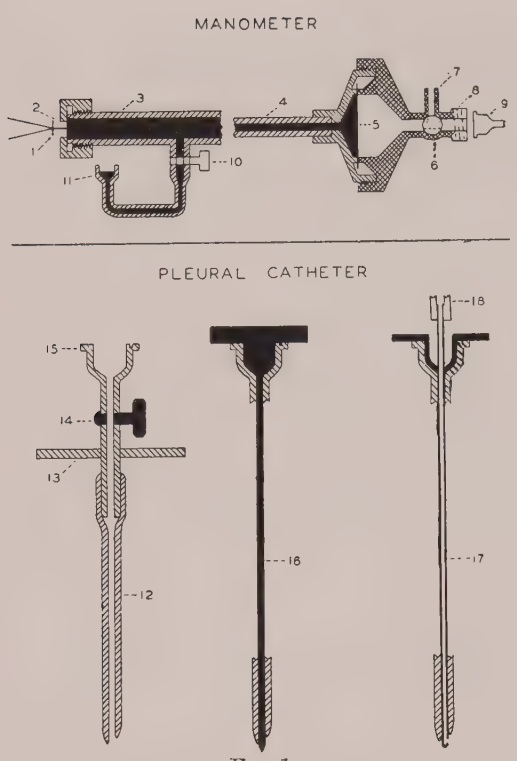

FIG. 1.
Diagrammatic Sketch of the Intrathoracic Pressure Recording System

1. Mirror. 2; Front membrane; 3. Capsule; 4. Lead tubing; 5. Rear membrane; 6. Three-way stopcock; 7. Side arm to calibrating mercury manometer; 8. Adaptor for pleural catheter; 9. Taper of pleural catheter; 10. Side-arm valve in capsule; 11. Water reservoir; 12. Pleural catheter; 13. Flanged metal joint; 14. Valve; 15. Taper of catheter; 16. Solid stylet; 17. Hollow stylet; 18. Connection to water manometer of pneumothorax apparatus.

the intrathoracic pressure is highly desirable. This is particularly true in the investigation of the circulation through the right heart under conditions of unusual respiratory effort, or in the presence of abnormalities of the pulmonary circulation.

With the method described below, continuous records of the intrathoracic pressure cycle, synchronous with the records of the right intracardiac pressure pulses, have been obtained in 6 cases. In most of these, the femoral arterial pressure was simultaneously registered. The electrocardiogram was routinely taken as a part of the study in all instances. The technic employed for recording the intrathoracic pressure was as follows:

A modification of the Hamilton manometer[2] was designed, which uses a wider mirror-bearing membrane to allow greater sensitivity. The fluid-filled lead tubing leading from the capsule is closed, at the end nearest to the patient, by a wide membrane of heat-treated beryllium copper, 0.0009 inches thick and 2.5 cm in diameter, the function of which is to prevent contamination of the pleura with fluid. The membrane is held in place by a knurled nut of conical shape, hollow within, which terminates in a 3-way stopcock. One opening in the stopcock is connected with a mercury manometer, to permit calibration. The other opening is adapted to fit a specially designed pleural catheter. Because of the effect of varying environmental temperatures upon the internal tension of this sealed system, a side-arm valve, connected to a small water reservoir, is inserted into the capsule to permit repeated equilibration with the atmospheric pressure.

The pleural catheter consists of a short segment of No. 9F cardiac catheter, with smooth rounded end and an opening of suitable size at the intrapleural extremity. The opposite end is fitted to a flanged metal joint, which contains a stopcock, and which is tapered to permit air-tight fitting to the manometer by a simple rotary motion of the flange. A sharp-pointed solid stylet is inserted into the saline-moistened catheter for penetration of the outer chest wall; this is then replaced by a blunt-ended hollow stylet for puncture of the pleura. The second stylet is connected to the water manometer of a pneumothorax apparatus for immediate detection of entry into the pleural space. As soon as this is accomplished, the stylet is withdrawn far enough to permit closing the valve in the catheter, after which it is completely withdrawn. The catheter is then attached to the recording manometer, and the valve opened. Further manipulation is governed by inspection of the motion of the recording light beam. With sufficient novocaine infiltration of the site of the chest puncture, the catheter has been left in place without undue discomfort

[2] Hamilton, W. F., Brewer, G., and Brotman, I., *Am. J. Physiol.*, 1934, **107**, 427.

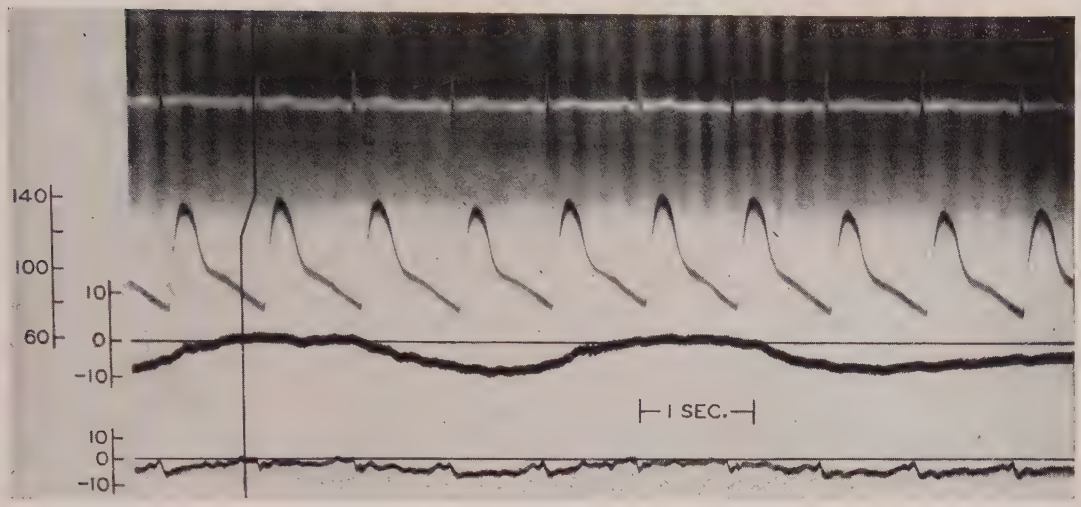

FIG. 2.

Femoral arterial pressure (upper curve), intrathoracic pressure (middle curve), and right intraauricular pressure (lower curve, retouched to improve contrast) from a patient with a recently-induced therapeutic pneumothorax. The respiration is somewhat deeper than normal. In this and succeeding figures, zero intracardiac pressure is taken as the hydrostatic level 5 cm below the angle of Louis. All scales represent mm of mercury.

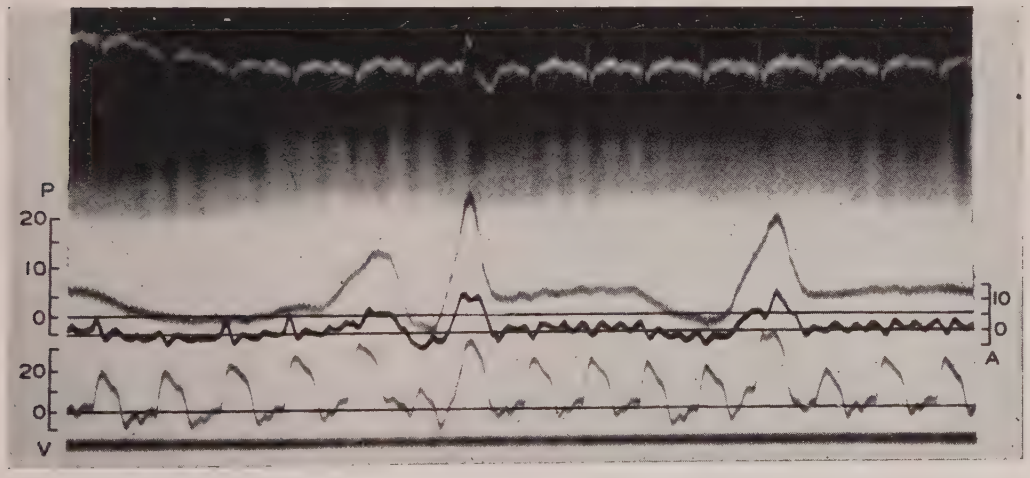

FIG. 3.

Intrathoracic pressure (upper curve), right intraauricular pressure (middle curve, retouched) and right intraventricular pressure (lower curve), from another patient with recent pneumothorax. The simultaneous recording of pressures from both chambers of the right heart was done through a double-lumen catheter.[3] The 3 sharp rises of intrathoracic pressure are due to 3 coughs.

for as long as 4 hours. The frequency of this recording system is about 25 vibrations per second, with a sensitivity of approximately 1.3 mm per mm of mercury pressure. The length of the optical lever is about 6 feet.

Summary. A method is presented for the optical recording of intra-pleural pressure

[3] Cournand, A., Bloomfield, R. A., and Lauson, H. D., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 73.

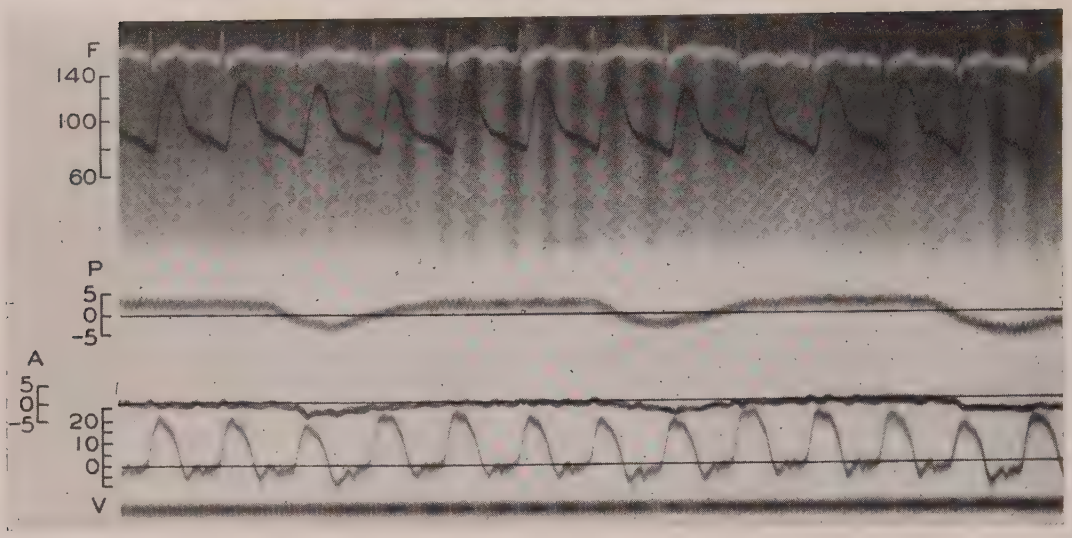

FIG. 4.

From above downward, femoral arterial, intrathoracic, right intraauricular (slightly damped) and right intraventricular pressure records from the same patient as in Fig. 3, recorded immediately after 500 cc of air had been injected into the intrapleural space. Respiration is quiet.

simultaneously with right heart pressure changes, utilizing the intracardiac catheterization technic in conjunction with a specially designed recording manometer and pleural catheter. Reproductions of several tracings are shown.

15097 P

Horizontal Intracortical Organization in the Cerebral Control of Limb Movement.*

R. W. SPERRY.†

From the Yerkes Laboratories of Primate Biology, Orange Park, Florida.

It has been widely assumed in theories of brain function that horizontal spread of excitations through the cortex is an important factor in cerebral integration. Not only the irradiation of discrete neural impulses through the fiber feltwork of the cortex, but also the mass conduction of intercellular electrical currents and potentials and the establishment of patterns of "field forces" spreading across the cortex have been presumed to play integrative roles of utmost importance in cerebral organization. The following experiments, however, undertaken to test the influence of such horizontal intracortical organization in the control of limb coordination have failed to demonstrate the existence of any major organizing influence of the sort. Lesions in the cortical arm areas of the monkey (Macaca mulatta) designed to disrupt any patterns of horizontal intracortical

* This work was carried out under contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Chicago.

† Research Fellow in Biology, Harvard University.

conduction either of discrete excitations or of mass electrical currents were found to cause only a slight, almost negligible, interference with motor coordination.

The lesions consisted of intersecting transverse and longitudinal cuts at intervals of about 2.4 mm made subpially with a thin knife and extending roughly through the depth of the cortex to the underlying medulla. Because the cuts filled immediately with blood, the effect of the operation was to partition the cortex by numerous vertical walls of clotted blood of about 0.08 mm thickness. The cuts interrupted the horizontal transmission of excitations through the cortex itself, but left intact the axon interconnections looping downward through the white matter. These incisions were placed throughout the exposed surface of the cortical arm region including areas 6, 4s, 4, 1, 2, 5, and 7 as represented on architectonic charts with some overlap into the neighboring trunk, leg, and face areas. As a control of the effects of these incisions the corresponding cortical area was destroyed completely by excision in other animals.

The control animals showed a severe paralysis of the contralateral arm and hand which persisted with only slow improvement for a month or longer. In contrast partitioning the area with vertical incisions as described above caused practically no disruption of arm coordination. On the first day after operation no difference was noted in the use of the affected and normal arms in rapid running and climbing movements in a large cage. Even in fine movements of the fingers and hands in manipulating pieces of food, the difference between the normal and affected limbs was very slight. A little weakness in the manipulative finger movements of the affected hand and some preference in such movements for use of the normal hand were the only symptoms noted on the first day after operation. By the seventh day no trace of any motor deficit could be detected. Nor were any sensory or autonomic effects apparent although no special effort was made to test for these. Uninstructed observers could not, even with close study, determine at this time which was the affected arm. Thus although the electrostimulable points in the cortex for movement of shoulder, elbow, wrist, and digits had been separated from each other by incisions as described above, there was no incoordination in the arm and hand movements. The same results were obtained in three unilateral cases. The effects were similar also when lesions of the same type were placed in the opposite hemispheres of these three animals about three weeks after the first operations. Histological examination of the cortex showed that the knife cuts had clearly effected a vertical partitioning of the grey matter as intended.

The surprising lack of disturbance in motor coordination caused by putting multiple vertical partitions in the cortical arm field casts evident doubt on hypotheses of cerebral function which have assumed that horizontal intracortical interaction between and within cortical areas is extensive and an essential element of cortical integration. The experiments indicate that functional interaction between cortical loci is achieved almost entirely by the systems of axons passing through the white matter and that the intracortical interaction *per se* is largely vertical. This is in accord with the descriptions of intracortical synaptic relations presented by Lorente de Nó[1] in which he has pointed out that intracortical connections are established chiefly in the vertical direction with the cells of the various horizontal layers linked together in vertical chains.

Further work is under way to determine the effects of making the incisions increasingly numerous and closer together and also at increased depths into the white matter. Extension of the experiments to the visual cortex has been started.

1 Lorente de Nó, Chap. XV in *Physiology of the Nervous System* by J. F. Fulton, Oxford University Press, N.Y., Inc., 1943.

15098

Further Report on Effect of Para-Aminobenzoic Acid in Experimental Tsutsugamushi Disease (Scrub Typhus).

E. S. Murray, C. J. D. Zarafonetis, and J. C. Snyder. (Introduced by S. Bayne-Jones.)

From the Cairo Unit of the United States of America Typhus Commission.

In a preliminary note experiments were described which indicated that the administration of the sodium salt of para-aminobenzoic acid (Na PAB) reduced the mortality of experimental tsutsugamushi disease (scrub typhus) in gerbilles.[1] Additional tests of Na PAB have now been completed with other strains of *Rickettsia orientalis*. The data of these further trials are presented below.

Description of the strains of R. orientalis. The sources of the Ceylon and Calcutta strains have been cited in another report.[2] The Karp strain[3] was obtained through the courtesy of the National Institute of Health, Bethesda, Maryland.* The inoculum was prepared from peritoneal fluid of infected gerbilles after one or more passages had been made in these rodents.

Method of administration of Na PAB in the diet. The plan of therapy as followed in Experiments 3 and 4 in the preliminary note, was changed slightly to improve the regularity of the food intake of the gerbilles. The changes were these: (1) water bottles were not used; (2) all of the food dishes were removed, cleaned, and filled with freshly prepared wet mash, and then replaced in the cages, every 8 hours during the 21 days of each test. The composition of the wet mash was essentially the same as previously described: 80 g of dried, powdered Egyptian bread, 2 g of sodium chloride, and 60 cc of milk constituted the basic mixture. For the controls 40 cc of tap water was added. For the treated gerbilles 40 cc of approximately 10.4% Na PAB solution was added. In most instances the food dishes were filled with slightly more of the mash than the gerbilles were able to consume in any 8-hour period. The control mash and the Na PAB mash were placed in the cages a few hours after the inoculation of *R. orientalis* suspensions in every test except Experiment 5. No attempt was made to determine the effect of delayed administration of Na PAB on the course of the infection.

Method of administration of Na PAB parenterally. All of the gerbilles received subcutaneous injections every 8 hours from the 5th or 6th to the 16th or 17th day after inoculation with *R. orientalis*. The control gerbilles received saline, approximately 0.1 cc per 25 g body weight. The treated gerbilles received solutions of Na PAB, approximately 0.1 cc per 25 g body weight, the solutions varied in concentration from 10.4 to 11.6% in the different tests. This plan of treatment, in which the oral intake was supplemented by parenteral therapy, is referred to hereafter as the "routine combined Na PAB therapy."

The Na PAB was prepared by the addition of 500 cc distilled water to 84 g of Na HCO_3 and 137 g of PABA. In some instances the resulting dark brown solution was evaporated to dryness at room temperature, and the powder thus obtained was weighed out for the preparation of the solution in distilled water. The concentrated solutions were adjusted to the desired strength by the addition

1 Snyder, J. C., and Zarafonetis, C. J. D., Report to the Director of the U.S.A. Typhus Commission. Submitted for publication 9 October, 1944. In press.

2 Zarafonetis, C. J. D., Report to the Director of the U.S.A. Typhus Commission. Proc. Soc. Exp. Biol. and Med., 1945, **59**, 113.

3 McLimans, W. F., Grant, C. W., and Gersh, I., Report No. 1 (from the Naval Medical Research Institute, Bethesda, Maryland), 4 October, 1944.

* The authors wish to thank Lt. Comdr. A. Yeomans for transporting the infected animals to the Cairo laboratory.

of distilled water before injection into gerbilles.

Severity of the inoculum. Complete titrations to establish the number of minimal lethal doses on the basis of a 50% end point were not carried out in these tests. A few dilutions of the test inoculum were made and from the results it was possible to estimate that the inocula contained more than a specified number of "certainly fatal doses." As used in our report, the expression "one certainly fatal dose" indicates the smallest dose which produced death of all of the gerbilles. This method of very rough estimation of severity of infective inocula has obvious deficiencies but it was the only practical method under the circumstances in which these experiments were performed.

Experiments 1, 2, 3, and 4 were described in the preliminary note.[1]

Experiment 5. Calcutta strain. Inoculum: 6 cc of pooled peritoneal washings obtained from 2 moribund, 13th passage gerbilles; 1 cc of this fluid was diluted to 100 cc in saline. Control group: 7 gerbilles received no Na PAB at any time. Treated gerbilles: 4 received "routine combined Na PAB therapy;" 3 received no Na PAB for the first 48 hours, but thereafter received the "routine combined Na PAB therapy." Titration: Dilutions in saline to $10^{-3.5}$, $10^{-4.9}$, and $10^{-5.3}$. Results in Table I.

Experiment 6. Ceylon strain. Inoculum: 7 cc of pooled peritoneal washings obtained from 3 moribund, 7th passage gerbilles. This was diluted with saline to 10^{-1}. One cc of this dilution was inoculated i.p. into 54 gerbilles as follows: 27 gerbilles on control diet and saline injections subcutaneously from 6th to 17th day; 27 gerbilles on "routine

TABLE I.
Experiment 5. Calcutta Strain.

	Controls		Treated	
Conc. of inoculum	Time of death of each gerbille, days after inoculation	Ratio survivors to total in group	Time of death of each gerbille, days after inoculation	Ratio survivors to total in group
10^{-2}	11, 11, 11, 12, 12, 12, 12	0/7	18	3/4
			16*	2/3*
$10^{-3.5}$	11, 12, 13, 13	0/4		
$10^{-4.9}$	12, 13, 13, 13	0/4		
$10^{-5.3}$	13, 13, 14	0/3		

The gerbilles weighed from 30 to 50 g at the time of inoculation; they were evenly distributed between control and treated groups according to their weights.

* No treatment for first 48 hours; thereafter, "routine combined Na PAB therapy."

TABLE II.
Experiment 6. Ceylon Strain.

	Controls		Treated	
Conc. of inoculum	Time of death of each gerbille, days after inoculation	Ratio survivors to total in group	Time of death of each gerbille days after inoculation	Ratio survivors to total in group
10^{-1}	9, 10, 10, 10, 10, 10, 10, 10, 10, 10, 11, 11, 11, 11, 11, 11, 11, 11, 11, 11, 11, 11, 11, 11, 12, 12, 12	0/31	19, *19*	29/31
$10^{-2.3}$	12, 12, 13, 13	0/4		
$10^{-3.6}$	14, 15, 15	1/4		
$10^{-4.9}$	14, 15, 15, 18	0/4		

The gerbilles weighed from 26 to 55 g at the time of inoculation; they were evenly distributed between control and treated groups according to their weights.

The italicized figure in the treated gerbille column indicates that the animal did not show characteristic findings of *R. orientalis* infection at autopsy.

combined Na PAB therapy." Titration of inoculum: dilutions in saline to $10^{-2.3}$, $10^{-3.6}$, and $10^{-4.9}$. One cc of each dilution inoculated into 4 gerbilles i.p. Results in Table II.

Experiment 10. Karp strain. Inoculum obtained from second passage in gerbilles of the material derived from white mice brought to Cairo from the National Institute of Health. Moribund gerbille sacrificed; 3 cc fluid from peritoneal cavity was lightly centrifuged for a few minutes; the supernatant constituted the "undiluted inoculum." This was diluted with saline to 10^{-1} and the latter was lightly centrifuged for a few minutes. Further tenfold dilutions were thereafter made without centrifuging. Broth cultures were negative. Treatment was "routine combined Na PAB therapy." All of the control gerbilles had characteristic autopsy findings. None of the 4 treated gerbilles which died had typical autopsy findings. Results in Table III.

Experiment 11. Karp strain. Inoculum obtained from second passage in gerbilles of the material derived from guinea pigs brought to Cairo from the National Institute of Health. Moribund gerbille sacrificed; 1 cc fluid obtained from peritoneal cavity; this was diluted with 3 cc saline and the resulting suspension was called "undiluted inoculum." Serial tenfold dilutions in saline were made. Broth cultures showed no growth. Treatment was "routine combined Na PAB therapy." All controls died with

TABLE III.
Experiment 10. Karp Strain.

	Controls		Treated	
Conc. of inoculum	Time of death of each gerbille, days after inoculation	Ratio survivors to total in group	Time of death of each gerbille days after inoculation	Ratio survivors to total in group
10^{-2}	8, 8, 9, 9, 10, 10, 10, 11, 12, 13	0/10		10/10
10^{-3}	11, 11, 11, 11, 11, 11, 12, 13, 13, 13	0/10	*11**	9/10
10^{-4}	12, 13, 14, 14, 14, 14, 15, 15, 15, 16	0/10	*16*†	8/9
10^{-5}	12, 15, 15, 17, 17	0/5	*13*,‡ *19*§	3/5
	Total	0/35	Total	30/34

The gerbilles weighed from 30 to 50 g at the time of inoculation; they were evenly distributed between control and treated groups according to their weights.

* Died in convulsion upon inoculation with R_x. Autopsy: chest filled with free blood; otherwise negative.

† Autopsy: minimal fluid in chest; no peritoneal exudate; liver and spleen only slightly enlarged.

‡ Autopsy: tremendous dilatation of gut; otherwise negative.

§ Autopsy: slight enlargement of liver and spleen; otherwise negative. The animals in this cage were sick for several days in the middle of the experiment, and the survivors improved.

TABLE IV.
Experiment 11. Karp Strain.

	Controls		Treated	
Conc. of inoculum	Time of death of each gerbille days after inoculation	Ratio survivors to total in group	Time of death of each gerbille, days after inoculation	Ratio survivors to total in group
10^{-2}	9, 9, 9, 9, 10	0/5	12, *15*	3/5
10^{-3}	9, 9, 9, 9, 10, 10, 10, 10, 10, 10, 10, 11, 11, 11	0/15	*13*, *18*, *19*	12/15
10^{-4}	14, 16, 16, 16, 16	0/5		5/5
10^{-5}	14, 14, 14, 14, 17	0/5		
	Total	0/30	Total	20/25

The gerbilles weighed from 30 to 50 g at the time of inoculation; they were evenly distributed between control and treated groups according to their weights.

The italicized figures in the treated gerbille column indicate that the animal did not show characteristic findings of *R. orientalis* infection at autopsy.

TABLE V.
Summary of Experiments 3,* 4,* 5, 6, 10, and 11, in Which Gerbilles Received "Routine Combined Na PAB Therapy."†

Conc. of inoculum	Controls		Na PAB treatment	
	Ratio survivors to total	% survivors	Ratio survivors to total	% survivors
"Undiluted"	0/8	0	2/8	25
10-1	0/38	0	34/39	87
10-2	0/31	0	25/30	83
10-3	2/33	6	28/32	88
10-4	0/15	0	13/14	93
10-5	0/5	0	3/5	60
Total, all inocula	2/130	1.5	105/128	82

* The protocols of Experiments 3 and 4 were reported in the preliminary note.[1]
† The table does not include the control gerbilles which received inocula for titration if such inocula were not also given to corresponding groups of treated gerbilles.

characteristic autopsy findings. Four of the 5 treated gerbilles which died did not have typical autopsies. Results in Table IV.

In the 8 different trials of PABA in gerbilles infected with the Ceylon, Calcutta, Imphal and Karp strains of *R. orientalis* there were two routines of administration of PABA. The first routine was the feeding of a dry diet containing 3% of PABA in the acid form; parenteral therapy either was not given, or was inadequate to produce sustained concentration of PABA in the blood. There were no survivors in the 2 experiments of this type (1 and 2),[1] and merely a slight prolongation of survival time of treated gerbilles as compared with the controls. The blood concentration of PABA by this plan of therapy is usually low and frequently zero.

The second routine of therapy in our experiments was the combination of oral and parenteral administration of the highly soluble sodium salt of PABA. This routine assured a sustained concentration of PABA in the blood of the treated rodents during the entire period of the tests. The results of the experiments in which this routine was adhered to throughout (No. 3, 4, 5, 6, 10, and 11), are summarized in Table V.

The striking difference between the mortality of the controls and the treated gerbilles is clearly evident. There were 130 control gerbilles and of these 2 survived (1.5%). There were 128 treated gerbilles and 105 survived (82%). The data in Table V were obtained from the inoculation of doses of *R. orientalis* which ranged in severity from more than "one certainly fatal dose" to more than a "thousand certainly fatal doses."

There were 3 additional experiments (7, 8, and 9) with the Karp strain in which the conditions were not comparable to those in the experiments just reported. A change in the plan of therapy was followed in all or part of 7, 8, and 9, as a consequence of which the control as well as the treated gerbilles did not eat well during part of the tests and some were observed to be sick before such signs could be ascribed to infection with *R. orientalis*. In 2 of the 3 experiments the inoculum was derived directly from mice rather than from gerbille passage material. The results were less satisfactory than those in Table V. A summary of all gerbilles in Experiments 7, 8, and 9 shows that one control gerbille survived of 79 inoculated (1.3%). Eighteen treated gerbilles survived of 80 inoculated (23%).

The difference between the results with the "routine combined Na PAB therapy" and the results with other routines of therapy serves to emphasize the importance of the method of administration of the substance in any attempt to evaluate its effect on the course of the infection. This may account for the relatively slight effect of PABA on experimental tsutsugamushi disease in white mice reported by Peterson and Fox[4] and by

[4] Peterson, O. L., and Fox, J. P., Report to the Director of the U.S.A. Typhus Commission, 28 November, 1944.

Grant and McLimans.[5] In both of these reports PABA was given only by mouth, in the acid form. In the experiments of Grant and McLimans the concentration of PABA in the diet was roughly one-tenth the amount which proved successful in our experiments. Indeed, it is surprising that any effect was noted with such a small amount of drug.

It is our opinion that a thorough clinical trial of PABA against tsutsugamushi disease in humans is justified by the definite effect which PABA exerts on experimental tsutsugamushi disease in gerbilles. This favorable effect has been fully verified by repeated trials, using strains of *R. orientalis* from widely separated regions (India, Ceylon, New Guinea). Furthermore, the clinical evidence from PABA therapy of epidemic louse-borne typhus[6] has shown that it is safe to give PABA to man in large amounts.

Summary. Additional evidence has been cited to indicate that the sodium salt of p-aminobenzoic acid definitely reduced the mortality of experimental tsutsugamushi disease in gerbilles. A description has been given of the routine of administration of Na PAB which was successful. Data from other, less successful, routines have been reported to emphasize the importance of the mode of therapy in the evaluation of PABA in experimental infection with *R. orientalis*. A clinical trial of PABA in human tsutsugamushi disease is strongly recommended.

5 Grant, C. W., and McLimans, W. F., Report No. 2 (from the Naval Medical Research Institute, Bethesda, Maryland), 11 November, 1944.

6 Yeomans, A., Snyder, J. C., Murray, E. S., Zarafonetis, C. J. D., and Ecke, R. S., *J. A. M. A.*, 1944, **126**, 349.

The authors acknowledge the valuable technical assistance of Sergeant J. Dworkowitz and Corporal D. Hogan.

15099

Certain Bacteriostatic Agents Added to Sera Used in Diagnostic Tests for Neurotropic Virus Infections.*

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During the past five years a large variety of serological tests for virus infections, usually *in vivo*, but sometimes *in vitro*, have been made in this laboratory on several thousand serum specimens. Sera were generally tested for mouse protection against the viruses of both Western equine and St. Louis encephalitis and many were tested against the Lansing strain of poliomyelitis virus (adapted to the mouse by Armstrong[1]). Occasionally tests were made against the Eastern equine encephalomyelitis virus, the Japanese B encephalitis virus, a recently isolated strain of virus from California with neurotropic tendencies[2] and, more rarely, against still other neurotropic viruses. In addition to these tests, many of the specimens were subjected to experimental *in vitro* tests.

* This investigation was carried out in collaboration with the Commission on Neurotropic Virus Diseases, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Division, Office of the Surgeon General, U. S. Army; and under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of California. Aided by a grant from the National Foundation for Infantile Paralysis.

1 Armstrong, C., *Pub. Health Rep.*, 1939, **54**, 1719.

2 Hammon, W. McD., and Reeves, W. C., unpublished data.

TABLE I.

Experiment 1. The Effect of Zephiran 1:10,000, Merthiolate 1:10,000, and Sodium Sulfathiazole 1:500, as Serum Preservatives, on the Virus of St. Louis Encephalitis Under the Conditions of a Neutralization Test.

Bacteriostatic agent	Virus dilution				
	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Test of 1-11-44					
Zephiran	6/6*	5/6	4/6	0/6	0/6
Merthiolate	5/6	5/6	4/6	0/6	1/6
Sod. Sulfathiazole	6/6	6/6	4/6	1/6	0/6
Control	6/6	6/6	3/6	1/6	0/6
Test of 2-1-44					
Zephiran	6/6	6/6	6/6	4/6	0/6
Merthiolate	6/6	6/6	6/6	4/6	1/6
Sod. Sulfathiazole†	3/3	4/4	4/4	1/3	1/5
Control	6/6	6/6	5/6	3/6	0/4

* The numerator indicates the number of mice which died and the denominator the number inoculated in this and the following tables.

† Solution of Sod. Sulfathiazole had become toxic from holding for 20 days and several mice developed convulsions and some died soon after inoculation.

Sera for the most part, have been stored at about 5° C. As a result of repeated handling, and sometimes careless handling, contaminated sera frequently have been encountered. This has happened often enough to make it imperative to find some bacteriostatic agent to control the contamination without inactivating the viruses employed. Several agents were tested before one was finally rather arbitrarily selected for regular use.

Methods and Materials. After the desired quantity of the bacteriostatic agent had been added to normal rabbit serum, the serum was mixed and "incubated" with a virus suspension exactly as it would be in the course of a routine neutralization test. When the study was begun the technic of the neutralization test was that described by Hammon and Izumi.[3] Tubes of serum and virus were held at 5° C for five hours before inoculation into mice. Later, when it was noted in earlier literature,[4] and confirmed in our laboratory, that more effective neutralization occurred in the intracerebral test after incubating immune serum-virus mixtures for 2 hours at 37° C, the sera containing the bacteriostatic agent was incubated with the virus in this way before inoculation.

Five or ten fold serial dilutions of each virus were prepared and the range of dilutions so planned that all mice in the controls would die after inoculation with at least one or two of the lower dilutions, and few or none would die after inoculation with the highest dilution. Ampoules of frozen mouse-brain-virus suspension were used which had been previously titrated under similar circumstances. The tubes containing the serum-bacteriostatic agent-virus mixture to be inoculated, each contained 0.2 ml of serum including the bacteriostatic agent at the stated dilution, and 0.2 ml of a virus suspension. Thus, the bacteriostatic agent, in one half of its previous concentration in serum, was left in contact with the virus for either 5 hours at 5° C, or 2 hours at 37° C, prior to inoculation. As a control, serum to which saline had been added in the same amount as the bacteriostatic agent in the other tubes was similarly tested with the same serial dilutions of virus.

Zephiran, Merthiolate and Sodium Sulfathiazole. Experiment 1 (St. Louis virus).

Two tests were made with the St. Louis virus using Zephiran (Alkyl dimethyl benzyl ammonium chloride) in a 1:10,000 final dilution in the serum, Merthiolate, 1:10,000 and sodium sulfathiazole, 1:500 (0.2 per cent). These mixtures were held for 5 hours at 5° C. Results are shown in Table I. It

[3] Hammon, W. McD., and Izumi, E., *J. Immunol.*, 1942, **43**, 149.

[4] Cox, H. P., and Olitsky, P. K., *J. Exp. Med.*, 1936, **64**, 217.

TABLE II.

Experiment 6. The Effect of Phenyl Mercuric Borate, 1:50,000, as a Serum Preservative on the Virus of St. Louis Encephalitis Under Conditions of a Neutralization Test.

Bacteriostatic agent	Virus dilution			
	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Test of 5-23-44				
Phenyl Mercuric Borate	6/6	6/6	3/6	0/6
Controls	6/6	5/6	3/6	0/6

TABLE III.

Experiment 7. The Effect of Phenyl Mercuric Borate, 1:50,000, as a Serum Preservative on the Virus of Western Equine Encephalitis Under Conditions of a Neutralization Test.

Bacteriostatic agent	Virus dilution			
	10^{-7}	5×10^{-7}	25×10^{-7}	125×10^{-7}
Test of 5-23-44				
Phenyl Mercuric Borate	6/6	4/6	1/6	0/6
Controls	4/5	5/6	1/6	1/6

TABLE IV.

Experiment 8. The Effect of Phenyl Mercuric Borate, 1:50,000, as a Serum Preservative on the Virus of Poliomyelitis (Lansing Strain Mouse-adapted) Under Conditions of a Neutralization Test.

Bacteriostatic agent	Virus dilution		
	25×10^{-1}	25×10^{-2}	25×10^{-3}
Test of 10-17-44			
Phenyl Mercuric Borate	10/10	6/10	1/10
Controls	9/10	7/10	2/10

will be noted that none of the agents used had any apparent effect upon the virus. Sodium sulfathiazole, however, was unstable in solution and became toxic after standing for a few days, for a number of the inoculated mice developed convulsions, and some died. Following this experience and that of Experiment No. 2, performed on the same day, sodium sulfathiazole was not used again.

Experiment 2 (Western equine virus)

Two tests were made against the Western equine virus with Zephiran, and one with Merthiolate and sodium sulfathiazole. These agents were used in the same concentrations in serum as in the previous experiment; Zephiran 1:10,000, Merthiolate 1:10,000 and sodium sulfathiazole 1:500. In the first experiment 5 fold dilutions were employed and in the second 10 fold dilutions. Results were similar to those obtained with the St. Louis virus and it was concluded that there was no significant effect upon the virus by any of these agents.

Mercuric Cyanide. Experiments 3 and 4 (St. Louis and Western equine viruses)

At the suggestion of Dr. Troy C. Daniels of the College of Pharmacy of the University of California mercuric cyanide was tested. A 1 per cent solution in saline was added to the serum to make a final dilution of 1:10,000. Two tests were made with the St. Louis virus, and two with the Western equine strain. The St. Louis virus was used in 10 fold dilutions and the Western equine in 5 fold dilutions. No significant difference was noted between the preservative and control groups, with either virus.

Experiment 5 (Poliomyelitis virus)

The effect of mercuric cyanide was next tested upon the poliomyelitis virus (Lansing strain). Ten fold virus dilutions from 2.5×10^{-1} through 2.5×10^{-4} were employed, and mercuric cyanide was added to the serum in a final concentration of 1:10,000. The virus-serum mixtures were incubated for 2 hours at 37° C. The mercuric cyanide had

no significant effect on the strain of poliomyelitis virus.

Phenyl Mercuric Borate. Experiment 6 (St. Louis virus)

The next drug to be tested was phenyl mercuric borate. It was used in a final dilution of 1:50,000 in serum. It was first prepared in a dilution of 1:2,500 in saline for addition to the serum. All mixtures were incubated for 2 hours at 37° C. It will be seen in Table II that the drug had no effect upon the virus of St. Louis encephalitis.

Experiment 7 (Western equine virus)

When phenyl mercuric borate was tested with the Western equine encephalomyelitis virus, no virucidal effect could be detected. The results of the test are presented in Table III.

Experiment 8 (Poliomyelitis virus)

Poliomyelitis virus (Lansing strain) was not demonstrably affected by phenyl mercuric borate after incubation for 2 hours at 37° C. The results of the test are presented in Table IV.

Routine use of Preservative. For a short period of time following the tests with mercuric cyanide 1:10,000, this chemical was added to all sera as they were received. It appeared to be perfectly satisfactory. However, as soon as the tests were completed with phenyl mercuric borate, this latter agent was arbitrarily chosen to be used. A 1:2,500 solution in saline was prepared which has been kept in stock in the laboratory. In practice, to any tube of serum to be stored, 1 drop of this 1:2,500 solution is added per estimated milliliter of serum. The sera are stored at about 5° C.

The solution has been taken into the field and added to many sera obtained from wild mammals, domestic mammals, wild birds and domestic fowl. Many of these were undoubtedly contaminated with bacteria before the phenyl mercuric borate was added, for they were frequently collected under very unsatisfactory conditions in the field. A supply of a 1:2,500 dilution of phenyl mercuric borate was sent to the Kern County General Hospital, from which our laboratory receives by mail a large number of serum specimens. In all, a number slightly in excess of 1,000 serum specimens have had phenyl mercuric borate added to them within the past 12 months. Among all these sera only 8 have been found unsuitable for use from the standpoint of bacterial contamination, even though many have been used repeatedly for many tests. This is in great contrast to our former experience, particularly in regard to specimens of blood collected from animals shot or caught in the field.

Sodium sulfathiazole has not been used because solutions of it are not stable. Zephiran was not employed for it was felt that it might possibly interfere with certain *in vitro* tests because of its effect upon surface tension. Merthiolate or mercuric cyanide might prove satisfactory, for they are stable, sufficiently soluble and in a short trial of the latter no contraindication was found. The final selection of phenyl mercuric borate was more or less arbitrary, but we were probably influenced somewhat by the fact that it is being used to such a large extent in the preservation of plasma and other biological products, and used in a higher dilution than the other agents. Mercuric cyanide has not had—to our knowledge—such an extensive previous application.

Although we have not tested the effect of these agents on other viruses of the same group, through controlled serial dilutions, we believe it is safe to conclude that in all probability any of these agents may be safely used in neutralization tests with the other viruses. A number of our sera which contain phenyl mercuric borate have been used in neutralization tests against the Japanese B virus, against the new California virus and against the Eastern equine virus. None has been found to show any protection.

We have not performed any complement fixation tests with the above mentioned viruses but with mumps-virus-antigen, complement fixation tests[5] have been made in our laboratory on a number of the sera to which phenyl mercuric borate had been added.

[5] Enders, J. F., and Cohen, S., PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 180.

There was no apparent interference. Further tests along this line are indicated.†

Summary and Conclusions. 1. Zephiran and merthiolate 1:10,000 and sodium sulfathiazole 1:500 in normal rabbit serum had no virucidal effect upon Western equine and St. Louis encephalitis viruses as determined through serial virus dilutions under the holding conditions and dilution of a standard neutralization test.

2. Mercuric cyanide 1:10,000 and phenyl mercuric borate 1:50,000 in serum under closely comparable conditions did not affect either of the above viruses or the Lansing mouse-adapted poliomyelitis virus.

3. Phenyl mercuric borate 1:50,000 has been used over a period of one year in over 1,000 serum specimens which have been subjected to diagnostic tests. There has been no apparent interference. Many of these sera have been used repeatedly in neutralization tests against several neurotropic viruses and in experimental *in vitro* tests including mumps complement fixation tests. This agent has greatly reduced difficulties previously encountered due to contamination of sera during collection and routine handling.

Phenyl mercuric borate is therefore recommended for routine use as a bacteriostatic agent for serum specimens to be used in *in vivo* diagnostic tests for the neurotropic viruses. Further experimental trial is required before it can be recommended for all types of *in vitro* tests.

† Since writing this approximately 300 sera containing phenyl mercuric borate have been tested for complement fixing antibodies to the Western equine or the Japanese B viruses. In these tests and several negative and positive controls with larger amounts of the preservative no interference was detected.

15100

Propagation of Theiler's GD-VII Mouse Virus in Tissue Culture.

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Some years ago, various attempts were made* to cultivate monkey poliomyelitis (MV) virus in fluid cultures of monkey olfactory bulbs and sympathetic ganglia. Although this work gave promise in so far as the survival of the tissue elements was concerned, the results were otherwise disappointing. It was decided, therefore, to undertake the cultivation of other neurotropic viruses, preferably ones that could be carried in mice, in order to gain experience that might be applicable, eventually, to the cultivation of human poliomyelitis. Jungeblut and Sanders[1] reported the propagation in mouse embryo brain cultures of the SK monkey poliomyelitis virus that had previously been transmitted to mice by intermediary passage through cotton rats. The culture medium used by them consisted essentially of ox serum ultrafiltrate. The present communication deals with the propagation of Theiler's GD-VII virus[2,†] of mouse encephalomyelitis under a wide variety of special conditions.

Materials and Methods. After the virus had been carried for 20 culture passages, during which time several different sets of con-

* Unpublished experiments carried out in association with Dr. Howard A. Howe and Dr. Geoffrey W. Rake.

1 Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1940, **72**, 407.

2 Theiler, M., *J. Exp. Med.*, 1937, **65**, 705; Theiler, M., and Gard, S., *J. Exp. Med.*, 1940, **72**, 49, 79.

† The virus was supplied through the courtesy of Dr. Max Theiler of the Laboratories of the International Health Division, The Rockefeller Foundation, New York.

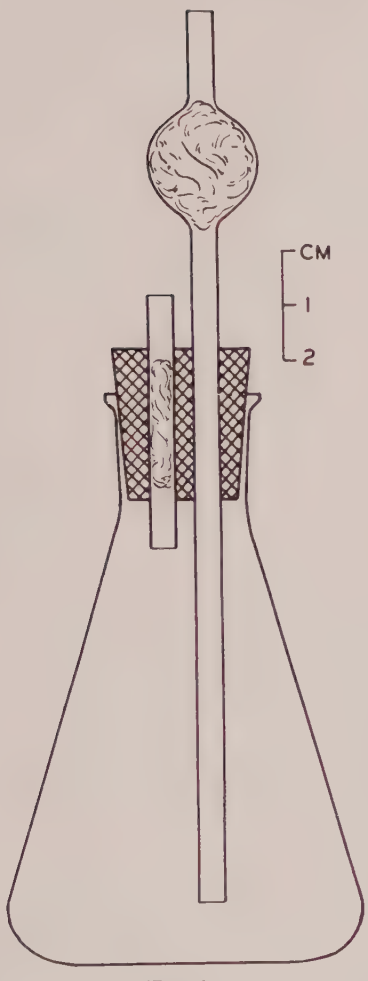

FIG. 1.

ditions were tested by comparative experiment, a routine set of procedures was chosen for all subsequent work. These procedures will now be outlined. For mouse embryo brain cultures, the supporting medium (3 cc) is prepared in 125 cc Erlenmeyer flasks and consists either of ox serum ultrafiltrate ($\frac{1}{3}$)[3,‡] or of heated (1 hr at 56° C) rabbit serum ($\frac{1}{3}$), combined with Simms' X7 balanced salt solution ($\frac{2}{3}$).[3] Before the tissue is added, the cell-free medium is adjusted to pH 7.1 or 7.2 by gassing for 1-2 min with a mixture of 8% CO_2, 21% O_2 and 71% N_2. For this purpose, the flasks are closed with 2-holed rubber stoppers each fitted with a long inlet tube surmounted by a cotton filter bulb and a short outlet tube partially filled with loose non-absorbent cotton (Fig. 1). After the tissue and the virus inoculum have been added, the special gassing device is replaced by solid rubber stoppers. As an indicator of pH, all cultures contain 0.001% phenol red.

The tissue component of the medium is prepared by mincing the brain on a glass plate. The mincing is done with cataract knives, with curved scissors, or with halves of razor blades held in hemostats. The tissues are chopped to extreme fineness. One large brain is required for each flask.

If the virus inoculum is prepared from brain material that has been preserved in 50% glycerine in saline, the tissues are chopped into coarse fragments and washed 3 times in great excess of glucosol (Tyrode's solution without bicarbonate). It is then ground in a mortar with quartz sand (60-80 mesh) and made up to 10^{-1} (1:10) with distilled water. This dilution is allowed to stand for 1 hour in an ice bath before the second dilution is prepared, as before, in distilled water. The third dilution (10^{-3}) and all higher dilutions are prepared in beef broth containing 10% serum ultrafiltrate. The 10^{-3} dilution is ordinarily used for the initial inoculation of the culture series; this and higher dilutions are used for mouse (Swiss strain) titrations to determine the potency of the original virus. For each culture, the inoculum is 0.3 cc; for each mouse, the intracerebral injection is 0.03 cc. Three mice are injected at each of several dilutions.

After 3-4 days incubation at 35° C, the culture virus is transferred to fresh brain cultures, and mouse titrations are made to test its killing power. Before the mouse titrations are made, and before the subcultures are infected (with 0.3 cc of undiluted culture material), the old culture mixture is centrifuged for 5-10 min at 2000 rpm in an angle head, in order to eliminate the tissue particles. Unlike the rabies virus cultivated under simi-

‡ The ox serum ultrafiltrate was supplied by William R. Warner & Co., Inc., New York, N.Y.

3 Simms, H. S., and Sanders, M., *Arch. Path.*, 1942, **33**, 619. See also: Sanders, M., and Molloy, E., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 327; Sanders, M., *J. Exp. Med.*, 1940, **71**, 113.

lar conditions,[4] the GD-VII virus is not so intimately bound to the culture tissues that it must be released by grinding.

Experiments and Results. The first experiment with the GD-VII virus was made with embryo mouse brain tissue and with ox serum ultrafiltrate as ⅓ of the medium (3 cc). This series gave positive mouse titrations through 15 culture passages (52 days), after which time it was lost as the result of bacterial contamination. But the culture virus did not kill mice in dilutions higher than 10^{-2} and 10^{-3}. The original virus inoculum was prepared from mouse brain material of the 129th mouse passage that had been preserved in glycerine for 135 days. The cultures were prepared in 125 cc Erlenmeyer flasks, were attached to a manifold in the incubator and gassed continuously with a mixture containing 8% CO_2; and, supernatant fluid from the unground cultures was used for serial transfers and for mouse titrations. Simultaneously, a sister series gave positive mouse titrations through 9 passages at dilutions of 10^{-4}. In this series, the culture medium (4 cc) consisted of ox serum ultrafiltrate (⅓) and Simms' salt mixture (⅔) and was prepared in 50 cc Erlenmeyer flasks that were incubated with closed stoppers after an initial pH adjustment with an appropriate gas mixture.

A series of 7 short experiments (none of more than 5 passages) was then made, but all gave negative results. In 4 of these experiments, the medium consisted partly of rabbit serum; in 3, both rabbit serum and ox serum ultrafiltrate were used in duplicate series of cultures, for purposes of comparison. In each of the 7 experiments, the original virus inoculum, whether from fresh mouse brains, from mouse brains preserved in glycerine, or from mouse brains kept frozen with CO_2-ice, killed mice in dilutions as high as 10^{-6}. In some of the experiments, the tissues were ground at transfer and for mouse titrations; in others, supernatant fluid from unground cultures was used. Certain of the experiments were gassed continuously; others were not. But the results were uniformly negative.

In a final effort to determine whether or not the original virus material was still capable of survival in cultures, a double series was inoculated with brain material that had been preserved for over 15 months in glycerine and that was still potent enough to kill mice at a dilution of 1:100,000 (10^{-5}). Half of the cultures were made with rabbit serum and half with serum ultrafiltrate. Again, the rabbit serum cultures gave negative results; but the serum ultrafiltrate cultures yielded virus of low potency that persisted through 5 passages. At this point, still another double experiment was started in which rabbit serum and ultrafiltrate were again used in two comparable series. This time, however, the virus inoculum was prepared from the fresh brain of a paralyzed mouse that had been inoculated with the 3rd culture passage material from the experiment just described. Here again, the ultrafiltrate cultures continued to support the virus, whereas the rabbit serum cultures became negative after the 3rd passage. In the ultrafiltrate cultures, the virus attained a potency of 10^{-5} in the 5th passage.

The culture virus that was developed in this experiment was carried eventually, and without intercurrent animal passage, for 20 transfers.§ During this time, it was used for 10 separate and distinct experiments, each of which was designed to compare the effect of two variations either in the composition of the medium or in other conditions that might affect the multiplication of the virus. It was also used in 3 experiments designed to determine the interval required for maximum yield. Over the entire period, it continued to multiply to the extent that, under favorable conditions, it would kill at least 2 out of 3 mice injected intracerebrally with unground cell-free culture fluid in dilutions as high as 1:1,000,000 (10^{-6}). The results of these

[4] Parker, R. C., and Hollander, A. J., PROC. SOC. EXP. BIOL. AND MED., 1945, **60**, 94.

§ Culture virus from the 10th passage was submitted to Dr. Theiler for verification of identity. On the basis of pathological findings and neutralization tests, Dr. Theiler concluded that the culture virus was indistinguishable from GD-VII.

TABLE I.
Theiler's GD-VII Mouse Virus in Fluid Cultures of Mouse Embryo Brain.*

Exp. No.	Series No.	Passages	Conditions under comparison	Temp. (°C)	Highest dilution of culture fluid that killed at least two of the three mice injected with each of the various dilutions tested at each successive passage							
					1st	2nd	3rd	4th	5th	6th	7th	8th
1	13233	1-5	R.S.†	37½	neg	neg	neg	neg	neg			
	13236		U.F.‡	”	10^{-1}	10^{-4}	10^{-2}	10^{-3}	10^{-2}			
2	13333	1-5	R.S.	”	10^{-1}	10^{-2}	neg	neg	neg			
	13336		U.F.	”	10^{-3}	10^{-4}	10^{-4}	10^{-4}	10^{-5}			
3	13437	6-13	37½°C		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-3}	10^{-3}	10^{-4}	—
	13441		35°C		10^{-4}	10^{-5}	10^{-5}	10^{-5}	10^{-6}	10^{-6}	10^{-6}	10^{-5}
4	13445	5-11	E-125§	”	10^{-3}	10^{-2}	10^{-3}	10^{-4}	10^{-4}	10^{-4}	10^{-5}‖	
	13449		E-50§	”	10^{-2}	10^{-2}	10^{-1}	10^{-3}	10^{-3}	10^{-3}	10^{-3}‖	
5	13606	12-16	Unground	35	10^{-6}	10^{-6}	10^{-5}	10^{-6}	10^{-6}			
	13610		Ground	”	10^{-6}	10^{-5}	10^{-5}	10^{-6}	10^{-6}			
6	13621	14-17	U.F.	”	10^{-5}	10^{-6}	10^{-6}	10^{-6}				
	13624		R.S.Filt.¶	”	10^{-4}	10^{-5}	10^{-5}	10^{-5}				
7	13710	14-17	R.S.	”	10^{-5}	10^{-5}	10^{-5}	10^{-5}				
	13714		R.S.Filt.	”	10^{-5}	10^{-5}	10^{-5}	10^{-6}				
8	13718	17-18	No gas**	”	10^{-6}	10^{-5}						
	13721		Gas††	”	10^{-5}	10^{-5}						
9	13823	18-20	U.F.	”	10^{-5}	10^{-5}	10^{-4}					
	13827		R.S.Filt.	”	10^{-6}	10^{-6}	10^{-5}					
10	13881	18-20	No gas	”	10^{-6}	10^{-5}	10^{-6}					
	13885		Gas	”	10^{-6}	10^{-5}	10^{-5}					

Exp. 1. Theiler's GD-VII virus (M.P. 129); from mouse brain in glycerine in 15½ months.
2. Virus from fresh brain of paralyzed mouse inoculated with 3rd culture passage material of 13236 series.
3. Culture virus from passage 5 of 13336 series; frozen in CO_2-ice 13 days.
4. Culture virus from passage 4 of 13336 series; frozen 17 days.
5. Culture virus from passage 11 of 13449 series. Virus from both series filtered through Pyrex (UF) fritted glass filters between 13th and 14th passages.
6. Culture virus from passage 13 of 13441 series.
7. Culture virus from passage 13 of 13606 series after filtration through Pyrex fritted glass filter; frozen 9 days.
8. Culture virus from passage 16 of 13606 series.
9. Culture virus from passage 17 of 13714 series.
10. Culture virus from passage 17 of 13710 series; frozen 12 days.

* Unless otherwise indicated, the medium (3 cc) consisted of ultrafiltrate of ox serum (1/3) and Simms' X7 solution; the flasks (Erlenmeyer, 125 cc) were tightly stoppered, without continuous gassing, during incubation; and, transfers of culture virus were made without grinding the tissue.

† Rabbit serum, unfiltered.

‡ Ultrafiltrate of ox serum.

§ Erlenmeyer flasks of 125 cc and 50 cc capacity.

‖ 35°C for final (11th) passage only.

¶ Rabbit serum, filtered.

** Preliminary pH adjustment with gas mixtures, but cultures left lightly stoppered during incubation.

†† Cultures gassed continuously.

experiments are presented in Tables I and II, and will now be summarized.

Serum Ultrafiltrate. Of the 10 experiments presented in Table I, 4 were designed to compare the effect of ultrafiltrate with that of heated rabbit serum when each was used as the main ingredient of the culture fluid. In 2 of these experiments (Exp. 6 and 9), the rabbit serum had been filtered through Pyrex (U.F.) fritted glass filters; in two (Exp. 1 and 2), the serum was unfiltered. In Exp. 1, the killing power of the ultrafiltrate cultures remained low; and at the end of the 5th passage the titer was reduced to 10^{-2}. But in Exp. 2, which was infected with virus from a mouse inoculated with 3rd culture passage material of the 1st experiment, the titer was increased to 10^{-5} by the end of the 5th passage. By this time, however, the sister series treated with rabbit serum gave negative results in dilutions of 1:10 and higher, just as the serum cultures of the first experiment had been negative throughout. In Exp. 6, the ultrafiltrate cultures improved slightly, al-

TABLE II.
Amount of GD-VII Virus Obtained After Various Intervals of Cultivation.

Exp. No.	Culture Nos.	Passage No.	Highest dilution of culture fluid that killed at least 2 of the 3 mice injected at 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}, respectively: After 1 day	After 2 days	After 3 days	After 4 days	After 6 days	After 7 days
I	13847-56	18	10^{-5}	10^{-6}	10^{-5}			
II	13889-96	18	10^{-5}	10^{-6}		10^{-5}	10^{-4}	10^{-4}
III	13905-12	19	10^{-5}	10^{-6}		10^{-5}		10^{-4}

though this increase was probably the result of incubation at 35° C, which had already been found to be superior to 37.5° C. In Exp. 9, this improvement was lost, and the killing power of the ultrafiltrate cultures was somewhat less than that of those containing filtered serum. It should be noted, however, that in 5 other experiments presented in Table I (Exp. 3, 4, 5, 8 and 10), serum ultrafiltrate was the main component of the culture fluid. And whenever, in these 5 experiments, the temperature of incubation was set at 35° C, the culture virus reached a potency of 10^{-5} and 10^{-6}.

Rabbit Serum. Before the experiments presented in Table I were undertaken, 7 short experiments made with rabbit serum had given negative results. And the first 2 serum experiments recorded in Table I were also negative. But in Exp. 6, 7, and 9, in which heated and filtered rabbit serum was used, the culture virus reached a potency of 10^{-5} and 10^{-6}. In Exp. 7, the advantages of filtration were shown to be negligible.

Temperature of Incubation. In the 3rd experiment presented in Table I, a comparison was made of the relative effect on the multiplication of the virus of incubating the cultures at 37.5° C and 35° C, respectively. The virus yield at 35° C was invariably higher than that obtained at 37.5° C; and this slight decrease in temperature is regarded as being responsible for the general improvement in the results obtained in all subsequent experiments.

Use of Gas Mixtures. From the results obtained in Exp. 8 and 10 (Table I), it will be seen that the virus content of cultures that were gassed continuously with a mixture of 8% CO_2, 21% O_2 and 71% N_2 showed no improvement over those in which the gas mixtures were used only to adjust the initial pH of the culture medium. The pH was maintained at a relatively constant level in each instance.

Effect of Grinding the Tissues. The fifth experiment presented in Table I was designed to compare the multiplication of virus in a double series inoculated with ground and unground culture material, respectively. In one series, the tissues were ground at transfer (and for mouse titrations) with Tenbroeck grinders that were kept chilled in an ice bath during the grinding. In the sister series, the material for the inoculation of the subcultures and for the mouse titrations was prepared from cell-free supernatant obtained by centrifuging the unground tissue-fluid mixture. Even after 5 passages, there was no appreciable difference in the amount of virus recovered from the two sets.

Later, after it had developed that the culture virus reached its maximum concentration much earlier than had been assumed, a comparative experiment was made with culture virus of the 22nd passage that had been frozen in the CO_2-ice box for 29 days before it was used to infect a series of 5 embryo mouse brain cultures. After 2 days' incubation, mouse titrations were made from the cell-free culture fluid after low speed centrifugation (5 min at 2000 rpm) of the unground material from the 5 cultures, and, simultaneously, a duplicate series of mouse titrations were made from the same culture material after the component tissues had been ground with Tenbroeck grinders, resuspended in the supernatant, and centrifuged again at low speed to eliminate tissue particles. The 2 sets of mouse titrations gave identical results (10^{-6}).

Size of Culture Flasks. Comparative series

were run in Erlenmeyer flasks of 125 cc and 50 cc capacity. In the larger flasks, 3 cc of medium were used, whereas 4 cc were used in the smaller flasks. The virus yield was definitely greater in the larger flasks. (Exp. 4, Table I).

Time Required for Maximum Yield. Three experiments were made in an effort to determine the length of time required to obtain the maximum concentration of virus in the culture medium. The virus used had been carried for 17 and 18 passages and was derived from the series shown in Table I. The culture medium consisted of serum ultrafiltrate and Simms' X7 solution; and the cultures were incubated at 35° C. Each experiment comprised 8 or 10 cultures. Mouse titrations were made after the 1st and 2nd day and at subsequent intervals up to 7 days. At the time of testing, 0.1 cc was removed from each culture; and the samples were pooled and diluted. The results are shown in Table II. In all 3 experiments, the greatest concentration of virus was obtained after 2 days' incubation, when the titer reached 10^{-6}. After 2 days, the concentration of culture virus diminished. But because the differences observed from day to day were not greater than tenfold, they were probably of doubtful significance.

Postembryonic Mouse Brain Cultures. Culture virus derived from 20th passage embryo mouse brain cultures (Exp. 10, Table I), and subsequently frozen at —60° C for 49 days, was propagated for 13 additional and successive passages in cultures of brain tissue from 1 day old mice (5 passages), 3 day old mice (3 passages), 5 day old mice (4 passages) and 14 day old mice (1 passage), respectively. At the end of the 5th passage in cultures prepared from the brains of 1 day old mice, the mouse titer was 10^{-6}. After 3 passages in cultures prepared from the brains of 3 day mice, the titer was reduced to 10^{-4}. In the next culture series, in which the brain tissue was obtained from 5 day mice, the titer increased to 10^{-5}. But in the final passage, with tissue from a 14 day old mouse brain, the titer dropped again to 10^{-3}. And at this point the experiments were discontinued. It seems likely, however, that the virus could have been propagated indefinitely and without loss of potency in cultures prepared from the day old brains.

Summary and Conclusions. (1) Theiler's GD-VII virus has been propagated for 20 passages (transfers) in cultures of chopped mouse embryo brain suspended in ox serum ultrafiltrate and Simms' X7 solution. After an initial series of culture passages, the cell-free culture fluid killed mice in dilutions as high as 1:1,000,000 when 0.03 cc was injected intracerebrally. Eventually, the same results were achieved when the ox serum ultrafiltrate was replaced by heated rabbit serum. (2) The virus multiplied more abundantly at 35° C than at 37.5° C; and, the yield was higher in 125 cc Erlenmeyer flasks containing 3 cc of medium than in 50 cc flasks containing 4 cc of medium. (3) No increase in virus output was ever obtained by inoculating subcultures with ground material; nor did grinding the culture tissues improve the mouse titrations. Also, no real advantage was gained by subjecting the cultures to continuous gassing with mixtures of O_2, CO_2 and N_2. (4) The concentration of virus in the culture medium was higher after 2 days' incubation than after 1 day. After 2 days, the yield diminished. (5) Culture virus from 20th passage embryo mouse brain cultures that had been frozen at —60° C for 49 days was propagated for 13 additional passages in cultures of brain tissue from 1 day old mice (5 passages), 3 day old mice (3 passages), 5 day old mice (4 passages) and 14 day old mice (1 passage), respectively.

15101

Propagation of Rabies Virus in Tissue Culture.*

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Over the past five years, several strains of rabies virus have been propagated for varying lengths of time in tissue culture in an effort to discover the conditions under which an increased concentration of this and other neurotropic viruses might be obtained. Previously, Kanazawa,[1] Webster and Clow,[2] and Kligler and Bernkopf[3] have reported the successful cultivation of rabies virus *in vitro*. Kanazawa employed rabbit embryo brain and Tyrode's solution without serum. Webster and Clow employed mouse or chick embryo brains together with Tyrode's solution containing 10% monkey serum, and were able to produce typical rabies in mice with 1/33,000 cc of the culture material. Kligler and Bernkopf reported that human or monkey serum seemed to be essential for the growth of the virus; when these were replaced by other sera, the virus failed to grow.

Materials and Methods. In the present study, use has been made of different strains of virus that have been carried for various periods in mice. The best culture results were obtained with a virus strain isolated in 1935 from a dog brain sent from Alabama and carried since then in the Connaught Laboratories through 106 intracerebral mouse passages.† Inasmuch, however, as this strain was used in only the more recent experiments, it is quite possible that the improved results may have been due in part, at least, to other factors.

Of the twelve series of culture experiments that were made, one series was carried through 57 passages, another series through 24 passages, one series through 19, one through 12, two through 10, and the others for shorter periods. Two passages of 3 and 4 days, respectively, were made each week. As a rule, the tissues were ground at transfer and for animal inoculation. In a few of the experiments, however, direct inoculations were made from culture to culture; and in these experiments the material for mouse titrations was made from cell-free supernatant obtained by centrifuging the unground tissue-fluid mixture.

All of the more recent experiments have been made in 125 cc Erlenmeyer flasks which, in certain instances, were fitted with stoppers accommodating inlet and outlet tubes[4] for continuous gassing with a mixture of oxygen (21 or 80%), carbon dioxide (8%) and nitrogen. In six of the earlier culture series a 2-armed, 5-cm Carrel flask was used and the cultures were gassed continuously through filter bulbs inserted in the rubber stoppers.[5]

Finely chopped embryo mouse brain was used as the tissue component of the medium, one large brain or its equivalent being used for each culture. In the early experiments, the fluid portion of the medium consisted either of heated (1 hr at 56° C) rabbit serum (¼ or ⅓) or of ox serum ultrafiltrate[6] (⅓), together with 1.4% sodium bicarbonate

* Part of the investigation covered in this report was carried out in the Biological Laboratories of E. R. Squibb & Sons, New Brunswick, N.J.

† This virus was provided through the courtesy of Dr. R. D. Defries and Mr. T. C. Campbell.

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2 Webster, L. T., *Rabies*, New York, Macmillan, 1942; Webster, L. T., and Clow, A. D., *Science*, 1936, **84**, 487; *J. Exp. Med.*, 1937, **66**, 125.

3 Bernkopf, H., and Kligler, I. J., *Brit. J. Exp. Path.*, 1937, **18**, 481; Kligler, I. J., and Bernkopf, H., *Brit. J. Exp. Path.*, 1938, **19**, 378; *Am. J. Hygiene, Sec. B*, 1941, **33**, 1.

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5 Parker, R. C., *Tissue Culture*, in Glasser, O., *Medical Physics*, Chicago, The Year Book Publishers, Inc., 1944, p. 1568.

6 Simms, H. S., and Sanders, M., *Arch. Path.*, 1942, **33**, 619. *Cf.* Sanders, M., *J. Exp. Med.*, 1940, **71**, 113; Sanders, M., and Molloy, E., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 327.

(1/6), and, to make up the balance, Tyrode's solution containing 4 times the usual amount of glucose. Because these mixtures tend to be quite alkaline, the pH was adjusted to 7.2, by means of a gas mixture, before the tissue was added, and thereafter, the cultures were gassed continuously, day and night. In the more recent experiments, the fluid medium consisted either of heated rabbit serum or ox serum ultrafiltrate (1/3) combined with Simms' X7 solution.[6] As before, the pH of the medium was adjusted before the tissues were added. As an indicator of pH, all cultures contained either 0.001% or 0.005% phenol red.

Squibb Strain of Fixed Virus. In the longest culture series, an early experiment of 57 passages, the titer was never unusually high, but the virus was retained throughout the entire period (28½ weeks). In the 41st passage, the material killed mice through 10^{-5}. Thereafter, the killing power of the virus dropped off until, near the end of the experiment, the mouse titer had fallen to 10^{-2}. In this series, 2-armed Carrel flasks were used with 4 cc of medium, which consisted of rabbit serum (1/4), 1.4% sodium bicarbonate (1/6), and Tyrode's solution containing extra glucose. The virus used was a Squibb fixed rabies strain in its 12th mouse passage; and the cultures were gassed continuously with a mixture containing 80% O_2, 8% CO_2 and 12% N_2.

Webster Strain. Three experiments, two of 7 passages and one of 8, were made with the Webster strain of virus‡ that had been passed through 126 mouse passages. In these experiments, 2-armed Carrel flasks were used with 3 cc of medium consisting of 25% rabbit serum, and the cultures were gassed continuously in the manner already described. The chopped brain tissue from two embryos was added to each culture. But at the termination of the experiments, the culture virus failed to kill mice in amounts less than 1/330,000 cc (0.03 cc of 10^{-4}).

Alabama Strain. The most satisfactory results were obtained in some of the more recent culture series in which the Alabama strain of virus was used. In the first of these experiments, the medium (3 cc) consisted of heated rabbit serum (1/3) and Simms' X7 solution (2/3). The original virus inoculum (1:1000), introduced as 1/10 of the medium, was prepared from a brain of the 109th mouse passage. The brain, which had been kept in a CO_2-ice box at —60° C for 32 days, was weighed, ground with quartz sand (60-80 mesh) and diluted 1:10 (10^{-1}) with distilled water. After it had been allowed to stand in an ice bath for 45 min, it was centrifuged 10 min at 2000 rpm and the clear supernatant was again diluted, 1:10) with glucosol (Tyrode's solution without bicarbonate) containing 10% heated rabbit serum for inoculation to the cultures. The cultures were prepared in 125 cc Erlenmeyer flasks and were incubated for 24 hours before the virus was added. The tissues were ground at transfer (and for mouse titrations) with Tenbroeck grinders that were kept chilled in an ice bath during the grinding. After low-speed centrifugation, the tissue was resuspended in the cell-free supernatant, and transferred in 0.03 cc amounts to the new cultures. At the 19th transfer (passage), the culture virus killed mice (Swiss strain) at a dilution of 10^{-6} (1:1,000,000). The original inoculum, titrated at the beginning of the experiment, killed four out of four mice that had been inoculated with virus at 10^{-7}. Unfortunately, higher dilutions were not tested. A separate pipette was used for each dilution.

Effect of Gassing and Cultures. An important feature of the 19-passage experiment just described was the attempt to determine the effect, if any, of continuous gassing upon the multiplication of the virus. For the first six passages, none of the cultures was gassed continuously. Thereafter, the series was broken into two parts, one of which was placed on the gas manifold for each successive passage, the other not. Comparative mouse titrations were made at the end of the 8th, 10th, 11th, 12th and 14th passages. Two of

‡ This virus was supplied through the courtesy of Dr. L. T. Webster, to whom the culture virus was eventually submitted for verification of identity. On the basis of complement fixation tests, Dr. Webster concluded that the culture virus behaved like typical rabies.

these tests showed the gassed cultures to be slightly better than the ungassed ones, but in the others the results were quite similar. Hence, it would seem that, for this particular virus, no real advantage is to be gained by supplying the tissues with extra oxygen provided, of course, that the buffering system in the medium is capable of maintaining the pH at an optimal level.

Effect of Grinding the Culture Tissues. Another feature of the 19-passage experiment was the titration in mice of culture virus prepared by grinding the tissues with quartz sand (60-80 mesh) and with the Tenbroeck grinders; and the results so obtained were compared with the results of mouse titrations made with the cell-free culture fluid after low speed centrifugation of the unground culture material. Tests made with culture material of the 15th and 19th passages showed grinding to be quite necessary in order to release the main bulk of the virus from the tissues, but there was little to choose between the two methods of grinding.

Rabbit Serum and Serum Ultrafiltrate. In another culture series, one of 12 passages, a comparison was made of the effect of heated (1 hr at 56° C) rabbit serum and ox serum ultrafiltrate when each of these were used as ⅓ of a medium, the balance of which consisted of Simms' X7 solution. From the beginning, the culture series was divided into two parts that were kept separate and distinct for each successive passage. The medium of one part of the series contained heated rabbit serum; the other, ultrafiltrate. Otherwise, the two halves of the experiment were comparable in every respect; and 3 cc of medium were used throughout. The virus inoculum was prepared from a mouse brain of the Alabama strain in the 109th mouse passage. The brain, which had been kept frozen in a dry ice box at —60° C for 5 days, was weighed, ground with quartz sand and made up to 10^{-1} with distilled water. After having been allowed to stand for 1 hour in an ice bath, the emulsion was centrifuged for 10 min at 2000 rpm and made up to 10^{-2} with distilled water. Subsequent 10-fold dilutions, up to and including 10^{-8}, were prepared in beef broth containing 10% horse serum (heated 1 hr at 56° C). Each culture was inoculated with 0.3 cc of the virus material at 10^{-3}. The potency of the original virus inoculum was determined by injecting mice intracerebrally with 0.03 cc of each of the higher dilutions. At 10^{-8}, one mouse of the three that were injected died with typical rabies. At each of the lower dilutions, all three mice succumbed. Throughout the experiment, the cultures were transferred twice a week at 3- and 4-day intervals, respectively. Also, the pH of the cultures was adjusted to pH 7.1 with an appropriate gas mixture and the flasks were closed with rubber stoppers before being set to incubate at 37° C.

Mouse titrations were made at each transfer. The greatest concentration of culture virus was obtained in the 6th passage. It was also in the 6th passage that the greatest difference was found to exist between the amount of virus contained in the rabbit serum cultures and in the ultrafiltrate cultures. In this passage, the rabbit serum cultures killed two out of three mice at 10^{-7} and all mice injected with lower dilutions. The ultrafiltrate cultures, on the other hand, failed to kill mice in dilutions higher than 10^{-4}. Beyond the 6th passage, the ultrafiltrate cultures improved considerably, just as the rabbit serum cultures showed a slight decrease in potency. And by the end of the experiment, it was not possible to detect any appreciable difference between the two sets. It should also be noted that the culture virus from both sets was frozen in a CO_2-ice cabinet for 11 days between the 5th and 6th passages.

Temperature of Incubation. Because it was found in a series of experiments[4] with Theiler's GD-VII mouse virus that virus production was more abundant at 35°C than at 37.5°C, a similar test was carried out with the rabies virus (Alabama strain). In an experiment of 9 passages, half of the cultures were carried at the one temperature, half at the other, and the results were determined by injecting mice with the ground culture material in dilutions ranging from 10^{-3} to 10^{-7}, inclusive. For three passages (2, 4 and 7), the virus yield was slightly greater at 37.5°C than at 35°C. For three passages (3, 5 and 8), the yield was slightly greater at 35°C. In two pas-

TABLE I.
Propagation of Rabies Virus in Cultures of Brain Tissue from Mice of Increasing Ages.

Passage	Culture Nos.	Tissue (age of mouse)	Mouse titer*	Passage	Culture Nos.	Tissue (age of mouse) days	Mouse titer*
1	13990-93	embryo	10^{-4}	13	14156-61	5	10^{-5}
2	13999-02	"	10^{-5}	14	14166-71	5	10^{-5}
3	14007-10	"	10^{-4}	15	14186-89	5	10^{-4}
4	14045-48	"	10^{-6}	16	14194-97	5	10^{-5}
5	14053-56	"	10^{-5}	17	14202-05	5	10^{-5}
6	14067-70	1 day	10^{-6}	18	14214-17	5	10^{-5}
7	14079-82	1 "	10^{-6}	19	14227-30	5	10^{-5}
8	14103-06	1 "	10^{-5}	20	14240-43	5	10^{-6}
9	14119-22	3 days	10^{-5}	21	14269-72	14	10^{-2}†
10	14137-39	3 "	10^{-5}	22	14302-05	14	10^{-4}
11	14141-44	5 "	10^{-5}	23	14316-18	14	10^{-4}
12	14145-50	5 "	10^{-5}	24	14324-27	14	10^{-1}

* Highest dilution of culture fluid that killed at least two of the three mice that were injected with each of the various dilutions tested.

† One of the three mice injected was killed both at 10^{-3} and at 10^{-4}.

sages (1 and 9), the results were identical at the two temperatures. And in the remaining passage, complete mouse titrations were not made. The cultures comprising the first 5 passages of the 24-passage culture series described in the final section of this report were the cultures that were carried at 37.5° C for the first 5 passages in this experiment.

Chick Embryo Brain Cultures. During the early part of the work, various unsuccessful attempts were made to cultivate the Squibb fixed virus in chick embryo brain cultures. The chick culture series were inoculated with virus that had been carried by intracerebral passage in mice and also with virus that had been carried for several passages in mouse embryo brain cultures. Unsuccessful attempts were also made to cultivate, in chick embryo brain cultures, mouse virus that had been adapted to chick tissue by serial intracerebral passage in embryonated eggs. To this end, the mouse virus was carried by the method of Dawson[7] through 23 chick embryo passages (187 days) during which time the virus continued to kill mice at dilutions of 10^{-3}. But when the virus was transferred to chick embryo brain cultures, it survived only a few passages.

Postembryonic Mouse Brain Cultures. Rabies virus (Alabama strain) derived from a mouse brain frozen at —60° C for 60 days was cultivated for 24 successive passages in cultures of brain tissue from embryo mice (5 passages), 1 day old mice (3 passages), 3 day old mice (2 passages), 5 day old mice (10 passages) and 14 day old mice (4 passages), respectively. The results are summarized in Table I. At the end of the 5th passage in the embryo brain cultures, the mouse titer was 10^{-5}. At the end of 20 passages, the last 10 of which consisted of brain cultures from 5 day old mice, the titer was still 10^{-5}. In this instance, then, the virus multiplied quite as readily in cultures prepared from the brains of 5 day old mice as in cultures prepared from embryo mice. In cultures prepared from the brains of 14 day old mice, the virus multiplied less abundantly than in the 5 day old brain cultures from which the virus was derived; and at the end of the 4th passage in cultures prepared from the older mice, the titer had dropped to 10^{-1}. At this point, the series was discontinued.

Before the 20th passage culture virus was transferred to cultures prepared from 14 day old mouse brains (Table I), two attempts were made to cultivate the virus in cultures prepared from the brains of 28 day old mice. Thus, one series of such cultures was inoculated with fresh culture virus that had been carried previously for 6 passages in cultures of brain tissue from 5 day old mice; another series, of three successive passages, was initiated with fresh culture virus that had been carried for 7 passages in cultures of the 5

7 Dawson, J. R., Jr., *Am. J. Path.*, 1941, **17**, 177.

day brains. But the results were negative throughout.

Finally, an attempt was made to discover whether embryo mouse brain culture virus could be cultivated directly in cultures prepared from the brains of 5 day old mice, or whether it was necessary to adapt the virus gradually by passing it through an intervening series of cultures prepared from the brains of 1 and 3 day old mice. Accordingly, virus from embryo mouse brain cultures and from brain cultures from 5 day mice were each transferred directly to sister cultures prepared from the brains of 5 day old mice that were litter mates. The mouse titrations made at the end of the experiment gave identical results. It would appear, therefore, that the intermediate culture passages were quite unnecessary as a means of adapting the embryo brain virus to brain cultures from 5 day mice.

Summary and Conclusions. (1) The rabies virus has been propagated for as long as 57 passages (transfers) in cultures of chopped mouse embryo brain suspended in rabbit serum and buffered salt solutions. Under conditions developed in shorter, more recent experiments, the virus multiplied to the extent that mice were killed regularly by the culture material in dilutions as high as 10^{-6} (1:1,000,000); and it was possible to produce typical rabies in mice with 1/33,000,000 cc of the culture virus. When ox serum ultrafiltrate was substituted for rabbit serum, the virus titer was somewhat less. (2) Comparative experiments showed the importance of grinding the culture material for mouse titrations. But no significant increase in virus output was ever obtained by subjecting the cultures to continuous gassing with mixtures of O_2, CO_2 and N_2. (3) A duplicate series of cultures incubated for 9 passages at 35° C and at 37.5° C, respectively, showed no consistent difference in the amount of virus produced. (4) Attempts to cultivate the rabies virus in chick embryo brain cultures were unsuccessful even when the virus had been adapted to chick tissue by serial intracerebral passage in embryonated eggs. (5) Rabies virus from a mouse brain frozen at —60° C for 60 days has been cultivated for 24 successive passages in cultures of brain tissue from embryo mice (5 passages), 1 day old mice (3 passages), 3 day old mice (2 passages), 5 day old mice (10 passages) and 14 day old mice (4 passages), respectively. Culture virus developed in brain material from 5 day old mice, failed to multiply in cultures of brain tissue from 48 day old mice.

15102

Age and Autonomic Balance.*

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It has been shown by Feldman, Cortell, and Gellhorn[1] that anoxia causes an excitation of autonomic centers leading to a discharge of the vago-insulin (v.i.) and sympathetico-adrenal (s.a.) systems. If the responsiveness of these centers to anoxia or similar procedures is taken as a standard the question may be investigated as to whether external or internal factors alter the reactivity of these structures, and thereby influence the central autonomic balance. The effect of temperature[2] and fever[3] and of changes in the endo-

* Aided by a grant from the Josiah Macy, Jr., Foundation.

1 Feldman, J., Cortell, R., and Gellhorn, E., *Am. J. Physiol.*, 1940, **131**, 281.

2 Gellhorn, E., and Feldman, J., *Am. J. Physiol.*, 1941, **133**, 670.

3 Feldman, J., and Gellhorn, E., *Endocrinology*, 1941, **29**, 141.

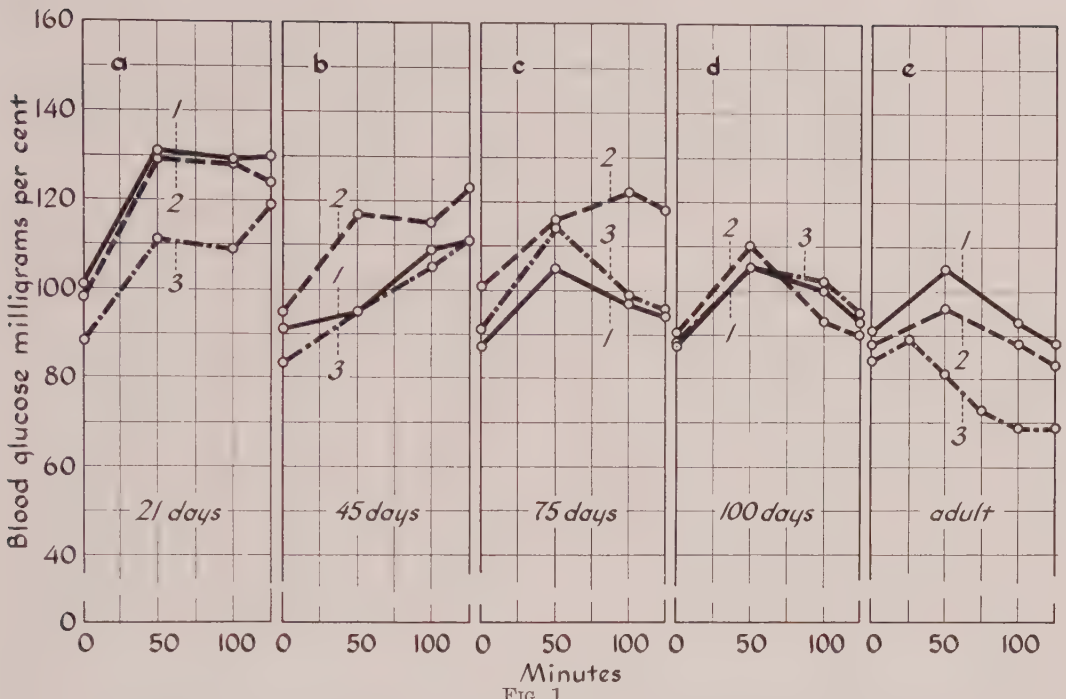

FIG. 1.

Influence of 5 periods of 25 min. each at 280 mm Hg on the blood sugar of rats at various age levels. Blood samples were taken after 50, 100, and 125 min. of anoxia. At each age level 3 groups of rats were used.

crine (thyroid)[4] and in the ionic balance,[5] has been reported previously. In view of the fact that experimental evidence seems to indicate a deviation in the reactivity of these centers in psychotic patients from that found in normal individuals (Gellhorn, Feldman, and Allen)[6] a better knowledge of internal physiological factors which have a central autonomic action appears desirable. The present investigation deals with the effect of age.

Methods. The experiments were performed on normal and adreno-demedullated rats at ages varying from 21 days to 1 to 2 years. After a fast of 22 hours the rats were subjected to a lowered barometric pressure of 280 mm Hg. for five periods of 25 minutes each. Blood sugar samples were taken before and after 50, 100, and 125 minutes exposure and analyzed using the Folin ferricyanide method as modified by Jeghers and Myers.[7] Three groups of rats, totaling between 14 and 25 animals were tested at each age level. In order to determine the reactivity of the v.i. system separately adreno-demedullated rats were tested at the age of 50 days and after they had become adults.

Results. Fig. 1 gives a graphic record of the results and demonstrates that age causes a progressive shift in the curves of hyperglycemia in rats subjected to the test period of lowered barometric pressure. The adult animals show at the beginning of the period of anoxia only a hyperglycemic phase which diminishes during the test period and gradually progresses into a state of hypoglycemia. Bearing in mind that hypoglycemia is a result of central stimulation of the v.i. system and that hyperglycemia is due to the excitation of the s.a. system,[1] the results indicate that in adult animals the s.a. system predominates at first and that later on the v.i. system comes into prominence. This method of repeated ex-

4 Gellhorn, E., and Feldman, J., *Endocrinology*, 1941, **29**, 467.

5 Gellhorn, E., and Feldman, J., *Am. J. Physiol.*, 1941, **134**, 603.

6 Gellhorn, E., Feldman, J., and Allen, A., *Arch. Neurol. and Psychiat.*, 1942, **47**, 234.

7 Jeghers, H. J., and Myers, V. C., *J. Lab. and Clin. Med.*, 1930, **15**, 982.

TABLE I.
Effect of Anoxia (5 Periods of 25′ each at 280 mm Hg) on the Blood Sugar (mg %) of Adrenodemedullated Rats.

Anoxia				
0′	50′	100′	125′	Remarks
104	82	79	83	Avg of 10 rats, 50 days
113	102	87	86	" " 8 " adults (more than 200 days)

posure to anoxia as first described by Van Middlesworth[8] is apparently a more effective activator of the v.i. system than the exposure of rats to 7% O_2 for 2 hours which was previously used in this laboratory. With the latter method the blood sugar was generally elevated at the end of the experimental period whereas with the present method a considerable number of rats showed a fall in blood sugar below the control level.

If these results are compared with those obtained on rats of 21 days of age it is obvious that the hyperglycemic phase is greatly accentuated in degree and duration in the young animals. In no instance was a fall in blood sugar below the control level observed, and in general, it was found that the rise in blood sugar observed after 50 minutes was maintained or even increased at the end of the experiment.

The experiments performed on rats at the at the age of 45 days were similar to those obtained at an earlier age. However, at the next stage (75 days) there is a definite change in the reactivity of the autonomic centers as disclosed by the blood sugar reaction in anoxia. In only one of the three groups is the reaction similar to that observed in younger animals. The other two groups (1 and 3) show only a temporary hyperglycemia which gradually diminishes in the later periods of anoxia. In rats at the age of 100 days this reaction approaches that seen in adult animals (older than six months). The latter show, however, as previously mentioned, a further reduction in duration and extent of the hyperglycemic phase and the appearance of hypoglycemia.

Since previous investigations showed that in experiments involving anoxia hyperglycemia depends on the s.a. and hypoglycemia on the v.i. system the progressive changes in the blood sugar curve discussed in this paper seem to indicate a shift in the reactivity of these centers. The results could be due either to a diminution in the reactivity of the s.a. system, an increase in the excitability of the v.i. system or a combination of these two factors. Experiments on adreno-demedullated rats which in anoxia react through the v.i. system only showed that the degree of hypoglycemia seen under these conditions differed not significantly between mature and immature rats (Table I). The maximal fall in blood sugar is almost the same in both groups although the v.i. effect starts earlier in the young animals. The difference between the two groups is, however, too slight to account for the effect of age seen in normal animals. The interpretation seems therefore warranted that in rats with progressing age the reactivity of the centers of the s.a. system diminishes whereas that of the v.i. system remains constant.

An attempt was made also to determine whether at very old age (rats older than two years) a further alteration in autonomic reactivity takes place. However rats of this age readily succumbed to the anoxia employed, apparently due to an insufficient adjustment of the vascular and respiratory systems.

Conclusion. When rats of varying age groups (21, 45, 75, 100, and more than 200 days) are exposed to five successive periods of anoxia (each lasting 25 minutes at 280 mm Hg.) the blood sugar curve shows a definite dependence on the age of the animal. Hyperglycemia is greatest and sustained in very young animals (21 and 45 days). In rats of 75 and 100 days the hyperglycemic phase is greatest initially and diminishes gradually as the experiment progresses. Finally, in adult animals the initial hyperglycemic phase is definitely lessened. At the end of the test

[8] Van Middlesworth, L., Kline, R. F., and Britton, S. W., *Am. J. Physiol.*, 1944, **140**, 474.

period the blood sugar is either about normal or definitely hypoglycemic as an indication that the vago-insulin system has come into prominence. This shift in the balance is, however, not due to an alteration in the activity of the vago-insulin system, but is a result of a diminished excitability of the sympathetico-adrenal system as age increases since the hypoglycemic effect of anoxia on the vago-insulin system as shown by experiments on adreno-demedullated rats is not influenced by the age of the animals.

15103 P

Effect of Glucose Injections on Fasting Ketonemia After Low Protein Diets.

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The rapid onset of a marked fasting ketosis following low protein diets has been attributed by MacKay *et al.*[1,2] to a lack of "stored" protein available for the formation of antiketogenic material during fasting. Their conclusions were based on the higher nitrogen excretion observed during fasting after the high protein intakes. The difference in nitrogen excretion between groups on 5 and 25% protein diets averaged approximately 6 mg per square decimeter of body surface per day during the first 48 hours. In a comparable experiment we observed a difference in excretion of 8 mg of nitrogen. It seemed unlikely that 30 mg of glucose per square decimeter of body surface, the amount available from the metabolism of protein containing 8 mg of nitrogen, would have an appreciable effect on the metabolism of the rat. However, Deuel[3] has reported a marked lowering of ketonuria after giving 100 mg of glucose per square decimeter of body surface daily.

The present study was designed to determine the effect of glucose available from the additional protein metabolized after higher protein intakes upon fasting ketonemia in the rat. Two groups of 12 male rats, weighing approximately 224 g, were fed for 3 weeks a 5% protein, high fat diet similar to that used previously[4] and then fasted for 48 hours. Twice daily during the fasting period one group received intraperitoneally a 5% glucose solution containing 15 mg of glucose per square decimeter of body surface. The other group received similar volumes of normal saline.

The blood ketone levels at the end of the 48-hour fast were 25.6 $\pm$ 1.3* mg % of acetone in the glucose injected animals and 28.5 $\pm$ 1.0 mg % for the controls. There was a slight lowering of the ketonemia by even this small amount of glucose but not to the level (18.8 $\pm$ 1.7 mg %) obtained on animals receiving a 25% protein diet. These results do not support the suggestion that the greater protein catabolism in the animals with the larger protein stores can account for sufficient antiketogenic material to prevent the marked fasting ketosis following low protein diets.

The available carbohydrate from the excess protein metabolized by the animals on the high protein diets does not appear to be the major factor responsible for the markedly decreased fasting ketosis in such animals and the effect must at least in part be otherwise explained. Other data we are accumulating suggests that an increased rate of metabolism in the animals on the low protein diets plays a more important role than the lack of protein stores.

[1] MacKay, E. M., Carne, H. O., Wick, A. N., and Visscher, F. E., *J. Biol. Chem.*, 1941, **141**, 889.

[2] MacKay, E. M., Visscher, F. E., and Wick, A. N., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 514.

[3] Deuel, H. J., Jr., Hallman, L. F., and Murray, S., *J. Biol. Chem.*, 1938, **124**, 385.

[4] Treadwell, C. R., King, W. C., Bebb, K. C., and Tidwell, H. C., *J. Biol. Chem.*, 1942, **143**, 203.

* Standard error of the mean included.

15104

Failure to Transmit Infectious Hepatitis to Chimpanzees.*

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The etiologic agent of infectious hepatitis is filtrable[1,2] and probably may be classified as a virus. It may be transmitted to man in serial passage[1] by feeding or parenteral inoculation, and to date man is apparently the only susceptible host. Unconfirmed reports have described the propagation of the agent of this disease in developing chick embryos,[3,4] canaries,[5] and pigs.[6] The general experience, however, has been a consistent failure to transmit this virus to incubating eggs and to a variety of small animals, including guinea pigs, white mice, rats, rabbits, hamsters, kittens, gerbils, baboons, rhesus monkeys, and other species such as *cercopithecus, erythrocebus,* and *colobus* monkeys.[7,8]

The present report describes an attempt to produce demonstrable disease in chimpanzees by the administration of materials known to contain an agent of infectious hepatitis. Two of the chimpanzees (No. 1 and 3)† were adults of 12-15 years of age, weighing from 65 to 70 lb. They had been in this country for several years. The other 4 animals (No. 4-7) were estimated to be between 2 and 3 years of age, weighing from 22-30 lb. They arrived from West Africa one month before the beginning of the experiment.

Infectious materials employed here were obtained from naturally occurring and experimentally induced human cases of infectious hepatitis. They are described in Table I.

TABLE I.
Source of Material.

Material	Material obtained from: Exp. case of infectious hepatitis	Material obtained from: Natural case of infectious hepatitis
Stool B		+8
" K	—9 —3	
" Bd	+2	
" Kh	—1 +2	
" Z	—1 +2	
Serums M	—4 —1 +1	
" K	—5	
" L	—5	
" G	—4	
" Mn	—5	

(—) indicates days before jaundice.
(+) indicates days after jaundice.
Multiple specimens from the same patient were pooled before administration.

Serum and stools were stored at dry-ice box temperature for periods ranging from 4-16 months. The serum was sterile and the stools contained no pathogenic bacteria before use.

The experiment consisted in administering

* These investigations were aided in part by the Commission on Neurotropic Virus Diseases, Board for the Investigation and Control of Influenza and Other Epidemic Diseases in the Army, Preventive Medicine Service, Office of the Surgeon General, United States Army.

Acknowledgment is made of the cooperation of the Department of Physiology, Yale University School of Medicine, in loaning one adult chimpanzee and providing technical assistance for this experiment.

1 Havens, W. P., Jr., Proc. Soc. Exp. Biol. and Med., 1945, **58**, 203.

2 Findlay, G. M., and Wilcox, R. R., *Lancet,* 1945, **1**, 212.

3 Siede, W., and Meding, G., *Klin. Wchnschr.,* 1941, **20**, 1065.

4 Siede, W., and Luz, K., *Klin. Wchnschr.,* 1943, **22**, 70.

5 Dresel, E. G., Meding, G., and Weineck, E., *Z. Immunitätsforsch u. Exp. Therap.,* 1943, **103**, 129.

6 Andersen, T. T., and Tulinius, S., *Acta M. Scand.,* 1938, **95**, 497.

7 van Rooyen, C. E., and Gordon, I., *J. Roy. Army M. Corps,* 1942, **79**, 213.

8 Unpublished observations of the authors.

† These 2 chimpanzees, No. 1 (Pop-Eye) and No. 3 (No-No), were obtained from the Department of Physiology, Yale University Medical School.

TABLE II.
Inoculum and Route of Administration of Infectious Materials.

Chimpanzee No.	Inoculum	Route
1	Stool B, K, Bd	I.N. and Oral.
3	Stool B, K, Bd, Z, Kh	I.N. " "
	Serum M, K	Par.
4	Stool Z	I.N. " "
	Serum L, G, Mn	Par. " "
5	Stool Z	I.N. " "
	Serum L, G, Mn	Par. " "
6	Stool Z	I.N. " "
	Serum L, G, Mn	Par. " "
7	Stool Z	I.N. " "
	Serum L, G, Mn	Par. " "

I.N.—Intranasally.
Par.—Parenterally.
Oral.—Orally.

these materials by different routes to 6 chimpanzees. The inoculum which each animal received is defined in Table II. Inoculation or feeding with these materials had produced infectious hepatitis in human volunteers.

Chimpanzees No. 1, 4, 5, 6 were observed for 4 months, chimpanzee No. 3 for 3 months and chimpanzee No. 7 for 65 days. Certain functions of the liver of each animal were determined before the administration of infectious material and every 7-10 days thereafter throughout the period of observation by the following tests: 1. the quantitative serum bilirubin determination,‡ 2. cephalin-cholesterol flocculation, 3. thymol turbidity,[10] 4. bromsulfalein dye retention. The adult animals were anesthetized with ether or ether and nembutal at the time of each testing; the young chimpanzees were handled without anesthesia.

All of the animals with the exception of No. 7 remained healthy throughout the period of observation. Tests of liver function in these 5 healthy animals were normal throughout the experiment with the exception of the cephalin flocculation which was positive in one adult consistently both before and after inoculation. Occasional falsely positive reactions to this test were encountered in the 3 young animals (No. 4, 5, 6) when an extremely sensitive antigen was used. The results of serial determinations of these various tests of liver function are recorded in Table III. It is of interest that in an equal number of determinations made contemporaneously in normal adult men by the same methods the average total serum bilirubin was 0.8 mg%, and the bromsulfalein dye retention was 5.4%.

TABLE III.
Results of Serial Determinations of 4 Tests of Liver Function in Chimpanzees.

Chimpanzee No.	No. of tests	Serum Bilirubin mg %			Bromsulfalein* dye % retained in 30 min.			Thymol turbidity units			Ceph. Floc. 24 hr
		Low	High	Avg	Low	High	Avg	Low	High	Avg	
1	16	.13	.34	.23	3	8	5.4	—	—	—	—
3	12	.20	.41	.28	2	5	3.	—	—	—	++++
4	16	.07	.34	.20	0	2	1.6	0	3	1	0
5	16	.07	.89	.29	0	4	1.9	0	3	1	0
6	16	.14	.62	.29	0	3	1.9	0	3	1	0
7	10	.13	.55	.33	4	16	12	2	8	4	++++

* 5 mg of bromsulfalein/kilo of body weight was injected intravenously.

Chimpanzee No. 7 was sick on arrival in the laboratory with a severe parasitic infestation with *Balantidium coli,* strongyloides, and hook worm. Both before and after inoculation there was intermittent slight increase of bromsulfalein dye retention, consistently positive cephalin flocculation, and occasionally positive thymol turbidity test. The serum bilirubin was normal throughout. This animal remained thin and weak, dying on the 65th day after inoculation. At autopsy there was a diffuse hemorrhagic pneumonia with sections of unidentifiable worms visible histologically in the lungs. The liver was essentially normal grossly and microscopically. The spleen showed numerous collections of hematin, suggestive of

‡ According to the method of Malloy and Evelyn[9] using the photoelectric colorimeter.

[9] Malloy, H. T., and Evelyn, K. A., *J. Biol. Chem.,* 1937, **119**, 481.

[10] Maclagan, N. F., *Nature,* 1944, **154**, 670.

chronic malarial infection, although no parasites had been found in smears of the peripheral blood made during life.

Chimpanzee No. 1 died of acute asphyxia following aspiration of vomitus during anesthesia at the end of the 4-month period of observation. Postmortem examination of the liver was normal grossly and histologically.

Failure to induce infective hepatitis in chimpanzees is in keeping with the failure to infect lower animals.

Summary. 1. Two adult and 4 young chimpanzees were given material known to contain the etiologic agent of infectious hepatitis. Five of the animals remained healthy and showed no evidence of impairment of certain tests of liver function. One animal which was sick before beginning the experiment, died of a severe parasitic infestation 65 days after inoculation. The liver was normal grossly and histologically. Another animal died accidentally at the end of the period of observation and the liver was also normal. 2. Data are presented on serial determinations of certain tests of liver function in the chimpanzee. Quantitative determinations of serum bilirubin and per cent of bromsulfalein dye retention were of a lower order than similar determinations in normal humans. The cephalin flocculation was consistently positive in one apparently healthy adult animal and in one young chimpanzee with an overwhelming parasitic infestation. Three young healthy animals had consistently negative cephalin flocculation tests.

15105

Effect of H Ion Concentration on Eluting Poliomyelitis Virus from Cotton Fiber.

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The present report describes an exploratory method in the elution of the Lansing strain of poliomyelitis virus from cotton swabs. These studies were carried out with a view toward finding a method of eluting virus from cotton swabs following their application to the oropharynx of patients with poliomyelitis.

Material and Methods. A pool (20% suspension) of Lansing virus was prepared using the brain stems and cords of mice that became paralyzed following intracerebral inoculation. Aliquots of this pool were stored in ampules at −70°C. The 50% mortality end-point was 1:3750 ± 1200.

Iso-osmolar phosphate and acetate buffers were made for the pH range 4.0 to 8.1. Dilutions of virus at the desired pH were prepared using 8 parts of normal salt solution and 2 parts of buffer. The Lansing virus suspension was diluted 1:100 and 1:500 at pH 4.0, 4.6, 5.0, 6.0, 7.0, and 8.0. In tests determining the effect of H ion concentration *per se* on mouse brain-virus suspension the menstrua were kept at room temperature for one hour and then inoculated into mice. In elution studies each virus-pH mixture was divided into two aliquots, *viz.*, (a) to 1.0 cc, 2 cotton pledgets (⅝″ surgical sponges) were added, and (b) to the remaining fraction no cotton was added. Following contact at room temperature for 1 hour the cotton sponges were removed and the eluate expressed into clean tubes, using 10 cc syringes. The pH of each solution was re-determined following inoculation of each pair of eluate and control virus suspensions. Slight change in the H ion concentration occurred (*viz.*, virus suspensions at pH 4.0 shifted to 4.2 and from pH 8.0 to 7.8).

Inbred mixed strains of colored mice 4 to 8

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TABLE I.

Infectivity of Eluate Obtained from Cotton Fiber Suspended in Diluent at Various pH's and Containing Lansing Virus. Fate of Mice Inoculated with (a) Virus-pH Diluent and (b) Eluate from Cotton Fiber Suspended in Virus-pH Diluent.

Dilution of virus	Inoculum	pH range											
		4.0		5.0		6.0		7.0		8.0		Mean totals	
		Deaths*											
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
1:100	Control (a)	29/31	93.7	22/30	73.5	28/30	93.5	21/24	87.5	28/32	87.6	128/147	87.0
,,	Eluate (b)	17/32	53.3	16/31	51.7	27/36	75.2	41/57	72.0	29/32	90.6	130/188	69.3
1:500	Control (a)	26/32	81.2	20/32	62.5	27/32	84.3	23/34	67.6	22/32	68.7	118/162	72.8
,,	Eluate (b)	8/31	25.8	10/30	33.3	6/29	20.6	17/56	30.3	17/32	53.1	58/178	32.5

* Surviving 24 hours after inoculation.

weeks of age, obtained from the Roscoe B. Jackson Memorial Laboratory, were used in these experiments. Following intracerebral inoculation mice were observed for 28 days.

The *mortality rate* in mice inoculated with mouse brain-virus mixture at different H ion concentration, and to which cotton may or may not have been added, has been used as an index of the presence of virus in 0.03 cc of inoculum. This index provides an estimation of the activity (and presumably the amount) of virus in the aliquot samples and in the cotton eluates at various H ion concentrations. At least 24 mice, and usually more, have been employed to determine significant differences in the mortality rates in these tests.

Results. A summation of the results found in 4 tests appears in Table I. The Lansing virus was eluted from cotton fiber best at pH 8.0. In 1:100 dilution of virus about 40% of the active infectious agent was removed from the eluate at pH 4.0, 22% at pH 5.0, and about 15% at pH 6.0 and 7.0. At pH 8.0 the amount of virus in the eluate is nearly of the same order as in the aliquot sample to which cotton was not added. Maximal removal of virus by cotton fiber took place in the pH zone (4.0 to 5.5) where flocculation was visible in the mouse-brain-virus suspensions.

The results obtained with 1:500 dilution of virus support and amplify those above, namely, that the amount of virus eluted from cotton fiber increases with rising pH. Maximal elution occurred at pH 8.0. In 1:500 dilution (5-10 MLD) sufficient virus was retained by the cotton fiber at pH 4.0 to 7.0 so as to reduce the virus content beyond the median lethal dose. That the removal of virus from cotton in this dilution is facilitated at pH 8.0 seems clear enough, but it is not possible to say that all available virus was recovered.

The effect of H ion concentration on the mortality rate of mice inoculated with Lansing virus (1:100 and 1:500 dilution) is summarized in Table II. In mice inoculated with virus at pH 4.6 the mortality rate is significantly higher than for other H ion concentrations which were tested. The survival time, particularly among those inoculated with 1:500

TABLE II.
Fate of Mice at Several pH-Virus Mixtures; 1:100 and 1:500 Dilutions of Lansing Virus.

Dilution of virus	pH of inoculum	No. mice inoculated*	No. mice dead	Median day deaths 24 hrs after inoc.	Mortality rate, %
1:100	4.0	85	77	6.6	90.5
"	4.6	59	58	5.5	98.2
"	5.0	88	76	6.1	86.3
"	6.0	81	74	7.8	91.3
"	7.0	70	60	7.5	85.7
"	8.0	79	65	7.5	82.2
1:500	4.0	56	47	9.0	84.0
"	4.6	52	50	3.2	96.1
"	5.0	85	64	8.1	75.2
"	6.0	51	45	10.5	88.2
"	7.0	93	73	9.7	78.4
"	8.0	61	46	9.5	75.4

* Surviving 24 hours after inoculation.

dilution of virus is markedly shortened. For pH values other than 4.6 the differences in mortality rates and survival time can be accounted for by chance.

Discussion. The results point out that the Lansing strain of poliomyelitis virus can be eluted best from cotton swabs at pH 8.0. However, the mortality rate at pH 8.0 (and also 5.0) in a large number of mice inoculated with virus at several pH's has been among the lowest in the series. There were between 16 and 21% fewer mice dying at pH 8.0 than at 4.6. The procedure suggested from the data is to elute virus from cotton at pH 8.0, and then readjust the inoculum to pH 4.6 (or 6.0) before testing in animals.

Hammon and Izumi[1] studied the effect of pH on the mortality rate in mice inoculated with Lansing virus. They observed a step-like increase in the mortality rate, and progressive narrowing of survival time with increasing H ion concentration. Our results have evolved using a relatively larger number of animals and more concentrated virus preparations than Hammon and Izumi used. The present data do not show a precise increase in mortality rate with increasing pH; however, Hammon's contentions were demonstrated in higher dilutions of virus than were employed in these experiments.

Summary. A method is reported for eluting the Lansing strain of poliomyelitis virus from cotton swabs. This virus is eluted best from cotton when the diluent is alkaline at pH 8.0. To obtain optimal mortality (morbidity) rates the eluate should be made acid (pH 4.6, etc.) before it is inoculated into mice.

[1] Hammon, W. M., and Izumi, E. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 579.

15106

Transmission of Streptomycin from Maternal Blood to the Fetal Circulation and the Amniotic Fluid.*

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Streptomycin, an antibiotic substance obtained from the mold *Actinomyces griseus*, supplements penicillin by arresting the growth of many of the gram negative bacilli. It is freely distributed in most body fluids follow-

* The work described in this report was done under a contract recommended by the Committee on Medical Research between the Office of Scientific Research and Development and the University of Pennsylvania.

TABLE I.
Concentration of Streptomycin in Maternal Blood, Cord Blood, and Amniotic Fluid Following Intravenous Injection of 250,000 Units.

Patient	wt., kg	Interval between injection and delivery		Streptomycin—units per cc		
		hr	min	Maternal blood	Cord blood	Amniotic fluid
O.T.	62	0	19	7	3	3
A.B.	75	0	40	9	2	1
B.C.	67	0	46	8	1	0
D.T.	72	1	0	13	4	5
M.C.	74	1	13	4	2.5	4
S.O.	77	1	27	12	8	5
H.C.	64	1	41	8	2	0
H.Cl.	66	2	30	10	5	1
D.R.	49	2	40	8	8	7
L.B.	65	2	52	4	2	2
C.R.	65	3	16	<1	<1	0
V.T.	80	5	18	1	<1	2
C.B.	52	8	33	<1	0	<1
G.R.	75	11	32	0	0	0

< = Less than.

ing parenteral administration, but it does not reach the spinal fluid very readily in the absence of inflammation, nor does it pass into or out of the alimentary tract readily.[1] It seemed worth while, therefore, to determine its concentrations in cord blood and amniotic fluid as a preliminary step to an evaluation of its usefulness in the treatment of intrauterine infections.

Experimental. Fourteen normal women at term in active labor were given a single intravenous injection of 250,000 units of streptomycin dissolved in 5.0 cc of normal saline. At the time of delivery a specimen of amniotic fluid was obtained from the gush of that fluid which usually accompanies the end of the second stage of labor. At the time the umbilical cord was cut, a specimen of placental cord blood was collected. Immediately thereafter a specimen of blood was withdrawn from the antecubital vein of the mother. Sterile oxalate tubes were used for all specimens. Streptomycin was assayed according to the method of Stebbins and Robinson.[2]

The injection time of the streptomycin solution was 2 minutes. All toxic reactions were subjective and transitory. As the drug was being administered, one patient asked if she were "going to sleep," one complained of "fullness in the head," two of "dizziness," and four of "headache." The remaining 6 patients volunteered no information and showed no signs of toxicity. There was no apparent toxic effect on the baby.

Table I shows: (1) streptomycin to be present in the cord blood and amniotic fluid 19 minutes after intravenous injection into the mother; (2) streptomycin concentrations in the cord blood and amniotic fluid to be generally less than half that found in the maternal blood; and (3) streptomycin concentrations to be very low in the specimens of maternal blood, cord blood, and amniotic fluid obtained more than 3 hours after injection.

Conclusion. Streptomycin appears in the cord blood and in the amniotic fluid following intravenous administration to the mother at term.

1 Zintel, H. A., Flippin, H. F., Nichols, A. C., Wiley, M. M., and Rhoads, J. E., *Am. J. Med. Sci.*, 1945, **210**, 421.

2 Stebbins, R. E., and Robinson, H. J., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 225.

15107

Intrasplenic Implantation of Multiple Pellets of Estrogenic Steroids.

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When pellets of estrone, α-estradiol, and α-estradiol-3-benzoate are implanted intrasplenically in spayed female rats either no estrus or a very short period of estrus results (Biskind and Mark,[1] Biskind[2]). On the basis of the results which follow subcutaneous transplantation of the spleen containing the pellet, it was believed that α-estradiol-3-benzoate is inactivated by the liver to a lesser extent than is α-estradiol (Biskind[2]). This difference is only apparent. Pellet implantation fails to take into account the many variations in peripheral activity (Segaloff[3]).

The present study was undertaken to ascertain whether the differences in the observations of Biskind and ourselves could be explained by employing multiple pellets and pellets diluted with cholesterol (Shimkin and White[4]). In addition, we have employed the implantation of multiple pellets in order to study those estrogens which are too insoluble for satisfactory intrasplenic injection studies.

Material and Methods. Young adult spayed female rats of the Sprague-Dawley strain were employed for this study. Pellets were prepared in a simple die and compressed by means of a hand arbor press. The pellets were 2 mm in diameter and weighed from 4.0 to 6.0 mg when implanted. When necessary transplantations of the spleen were performed according to our previously reported technic (Segaloff[3]) before implantation of pellets. The pellets were implanted in a previously prepared small pocket in the spleen which was then closed with a single stitch. Only one pellet was implanted at any one time. Additional pellets were implanted as necessary until either 28 continuous days of estrus were induced or a maximum of 10 pellets per animal had been implanted. Vaginal smears were made daily at approximately the same time from castration until the animals were sacrificed.

Results. The results are presented in Table I.

All of the estrogenic materials studied and listed in Table I were also implanted intrasplenically into spayed female rats with subcutaneous spleens divorced from the portal circulation. The data on these latter animals are not presented in tabular form since the pellets were absorbed at about the same rate as those in the spleen *in situ* and one pellet only of each compound either alone or diluted with 3 parts of cholesterol sufficed to produce the standard 28-day period of estrus.

From Table I it is apparent that a single pellet of ethinyl estradiol, estriol, α-estradiol-3-benzoate, or α-estradiol dipropionate sufficed to produce 28 continuous days of estrus even when implanted into the spleen *in situ*. Accordingly, pellets of these compounds diluted with 75% cholesterol were studied with the results seen in Table I. Only minute amounts appear to be absorbed from the estrogen-cholesterol pellets. For the purpose of tabulation, the assumption was made that all of the loss in weight was due to the absorption of the estrogen. The assumption is not wholly valid. Fuenzalida[5] has observed that in pellets of steroid in combination with cholesterol, the absorption is not selective, and cholesterol is absorbed as well as steroid. Thus, from the data on estrogen-cholesterol pellets we must conclude that ethinyl estradiol, estriol, α-estradiol-3-benzoate, and α-estradiol

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1 Biskind, G. R., and Mark, J., *Bull. Johns Hopkins Hosp.*, 1939, **65**, 212.

2 Biskind, G. R., *Endocrinol.*, 1941, **28**, 894.

3 Segaloff, A., *Endocrinol.*, 1943, **33**, 209.

4 Shimkin, M. B., and White, J., *Endocrinol.*, 1941, **29**, 1020.

5 Fuenzalida, F., *Rev. Canad. de Biol.*, 1944, **3**, 366.

TABLE I.
The Response of Spayed Female Rats to Intrasplenic Estrogen Pellets.

	No. of rats	Avg No. pellets	Avg abs./ pellet/day μg	Avg total abs. per/day for 28 days estrus μg
1. Ethinyl estradiol	5	1	99	99
25% Ethinyl estradiol, 75% cholesterol	5	3.8	2	7
2. Estriol	4	1	26	26
25% Estriol, 75% cholesterol	4	5.3	2	9
3. α-Estradiol-3-benzoate	5	1	13	13
25% α-Estradiol-3-benzoate, 75% cholesterol	5	7.3	2	13
4. α-Estradiol dipropionate	5	1	39	39
25% α-Estradiol dipropionate, 75% cholesterol	5	4.2	4	17
5. Estrone-3-benzoate	5	4.2	6	26
6. α-Estradiol	6	2	17	35
7. Equilin	3	2.3	20	47
8. Estrone	5*	6	11	68
9. Equilenin	4	6.5	24	156

* One animal failed to display 28 days of estrus even after 10 pellets; this animal is omitted from the summary.

dipropionate are extremely active when administered by the intrasplenic implantation of pellets containing cholesterol, but due to the minute amounts absorbed no conclusion can be drawn as to their relative activity.

The remaining estrogens can be placed in the following descending order of activity as intrasplenically implanted pellets of the crystalline estrogen. Estrone-3-benzoate > α-estradiol > equilin > estrone > equilenin.

Discussion. The data presented confirms the reports that the intrasplenic implantation of a single pellet of many estrogens does not suffice to produce a prolonged period of vaginal estrus. However, the above data on the multiple implantations of pellets would indicate that the production of prolonged estrus is a function of the amount of estrogen absorbed and the character of the estrogen. This is in agreement with the results obtained with intrasplenic injections. Unfortunately, as previously pointed out, the method of pellet implantation in the subcutaneous spleen is too crude a method to enable one to take into account the obvious differences in the peripheral activity of such estrogenic compounds.

In addition, it is important to note that estriol which was not soluble enough for adequate study by intrasplenic injection, is but slightly inactivated by passage through the portal system. Equilenin, on the other hand, which was also not sufficiently soluble for adequate study by intrasplenic injection, apparently is inactivated in the liver to a marked degree.

Summary. If a sufficient number of estrogen pellets are implanted intrasplenically in spayed female rats, prolonged periods of estrous are produced. The number of pellets required depends upon the relative activity of the estrogen and its rate of absorption.

We would like to thank: Dr. Ernst Oppenheimer of Ciba Pharmaceutical Products, Incorporated, for α-estradiol, α-estradiol-3-benzoate, α-estradiol dipropionate, ethinyl estradiol, and estrone-3-benzoate; Drs. D. A. McGinty and O. Kamm of Parke, Davis & Company for estrone and equilenin; Dr. O. Wintersteiner of the Squibb Institute for Medical Research for equilin. We would also like to thank Dr. W. O. Nelson for advice, encouragement and the use of his laboratory.

15108

Serologic Distinctness of Eastern, Western, and Venezuelan Equine Encephalomyelitis Viruses.*

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Infections produced in animals by the viruses of eastern, western, and Venezuelan equine encephalomyelitis are so similar in their symptoms and course that they constitute, from a practical standpoint, a single clinical entity.

Nevertheless, cross-neutralization and cross-protection tests have, on the whole, been rather consistent in showing that these viruses are immunologically distinct. A possible relationship, however, was suggested by Gallo and Vogelsang,[1] who found that eastern and western equine encephalomyelitis immune sera exerted a slight neutralizing action against the Venezuelan virus, and by Soriano Lleras and Figueroa[2] who, using a Colombian virus presumably of the Venezeulan type,[3] reported similar results.

On the other hand, the results of cross-immunity experiments have been at variance with interpretations derived from neutralization tests. Records and Vawter[4] reported that 6 of 8 horses immune to western equine encephalomyelitis virus resisted cerebral or nasal inoculation with the eastern virus. Meyer, Wood, Haring, and Howitt[5] reported that 12 horses hyperimmunized to western equine encephalomyelitis virus strains resisted intracutaneous inoculation of the eastern virus, and 1 of 3 other animals survived cerebral inoculation of eastern virus; 2 horses hyperimmunized to the eastern virus resisted cerebral injection of western virus. Howitt[6] found that 40% of 115 guinea pigs immune to the western virus were resistant to inoculation with the eastern virus. Beck and Wyckoff[7] observed that while guinea pigs immune to the western virus were fully susceptible to inoculation with the Venezuelan virus, animals immune to either the eastern or Venezuelan viruses showed some resistance to inoculation with the heterologous virus, outliving the controls by a day or more; the results were considered as suggestive of some relationship between the eastern and Venezuelan viruses. Carneiro and Cunha[8] reported that guinea pigs solidly immune to the Venezuelan virus were afforded a small amount of protection to inoculation with a Brazilian (eastern type) virus, and guinea pigs immune to the eastern virus regularly resisted inoculation with the Brazilian virus; in the reverse direction, animals immune to the Brazilian virus were fully susceptible to the Venezuelan virus and only partially resistant to the eastern virus.

In view of the relationships suggested by cross-immunity tests, it was considered not impossible that the failure of neutralization tests to substantiate the results of cross-immunity experiments may have been due to the severity and lack of sensitivity of the usual intracerebral neutralization technics. Since the extraneural neutralization test con-

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1 Gallo, P., and Vogelsang, E. G., *Rev. Med. Vet. y Parasitol., Caracas,* 1940, **2**, 143.

2 Soriano Lleras, A., and Figueroa, L., *Bol. Inst. Nac. Hig. Samper Martinez,* 1942, No. 8, 3.

3 Kubes, V., *Rev. Med. Vet. (Bogota),* 1943, **12**, 108.

4 Records, E., and Vawter, L. R., *J. Am. Vet. Med. Assn.,* 1934, n. s. **38**, 89.

5 Meyer, K. F., Wood, F., Haring, C. M., and Howitt, B. F., PROC. SOC. EXP. BIOL. AND MED., 1934, **32**, 89.

6 Howitt, B. F., *J. Immunol.,* 1935, **29**, 319.

7 Beck, C. E., and Wyckoff, R. W. G., *Science,* 1938, **88**, 530.

8 Carneiro, V., and Cunha, R., *Arq. Inst. Biologicb, São Paulo,* 1943, **14**, 157.

stitutes a much more highly sensitive method for the detection of antibodies to these viruses than does the intracerebral test,[9,10] this technic was used to restudy the possibility that serologic relationships exist between these viruses. Because of the impression gained in previous work[10] that the amount of virus neutralized by an immune serum tended to be somewhat greater in 3-day-old mice than in older animals, serum-virus mixtures were tested by the subcutaneous route in animals of this age.

Material and Methods. Viruses. The strains used were those employed in previous work.[10,11]

Fresh mouse brain virus was used for the neutralization tests. Mice were inoculated intracerebrally with 0.03 ml of source virus diluted to the desired concentration with nutrient broth containing 10% of normal sheep serum. When signs of infection appeared, the animals were killed with chloroform. The brains were removed, cultured, and stored overnight in the freezing compartment of a refrigerator. The next day 5 bacteriologically sterile brains were weighed, triturated without abrasive, and made into a 20% suspension, by weight, in 10% serum-broth. The suspension was centrifuged for 15 minutes at 1500 r.p.m. in an International centrifuge equipped with an angle head. The supernatant fluid was drawn off and used to prepare a series of tenfold dilutions in serum-broth; dilutions were always made by serial transfer of 0.5 ml amounts into 4.5 ml amounts of diluent.

Sera. Immune sera were prepared in rabbits. The animals were bled at weekly intervals until a large amount of normal serum had been accumulated from each; the serum specimens were then pooled according to animals, filtered through a Seitz EK pad, and stored at 4° without preservative.

Pairs of rabbits were immunized against each virus. For the eastern, western, and French neurotropic yellow fever viruses the following method was used. Two freshly removed infectious mouse brains were triturated in a mortar with gradual addition of 10 ml of 0.85% salt solution, and the whole of the resulting suspension (uncentrifuged) was injected intraperitoneally into a rabbit. The animals were bled out 10 days later, and the serum was filtered and stored.

Immunization with the Venezuelan virus necessitated the use of inactive material because of the high virulence of this virus for rabbits.[12] The animals were given a total of 4 intraperitoneal injections consisting of 5 ml of formalized mouse brain vaccine[10] weekly; after a rest period of 2 weeks, 5 ml of a 10^{-4} suspension in saline of active mouse brain virus was given subcutaneously. The animals were bled out 10 days later, and the serum was filtered and stored in the same manner as the specimens mentioned above.

Neutralization Tests. In order more accurately to evaluate the results obtained with an immune serum should cross-neutralization occur, normal serum taken from the animal furnishing the immune serum was always included in the same test.

Undiluted sera were distributed into 75 x 12 mm tubes, and an equal volume of virus dilution was added. Virus was added to the normal serum first and then to the immune serum, beginning with the lowest virus dilution and ending with the highest. Homologous serum-virus mixtures were prepared last and inoculated first. The tubes were shaken to mix the contents, which were inoculated without incubation into 3-day-old mice in 0.03 ml amounts; the inoculum was administered subcutaneously between the shoulders, the needle being inserted parallel to the vertebral column and withdrawn slowly during the injection. Six mice were used for each serum-virus mixture.

The serum-virus mixtures were kept on the table in a container of ice and water during the inoculations.

In each experiment immune sera to each of the 3 equine encephalomyelitis viruses were tested against one of the viruses, and serum from a rabbit immunized against the

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10 Lennette, E. H., and Koprowski, H., *J. Immunol.*, 1944, **49**, 375.

11 Lennette, E. H., and Koprowski, H., *J. Immunol.*, 1944, **49**, 175.

12 Lennette, E. H., and Koprowski, H., *J. Am. Med. Assn.*, 1943, **123**, 1088.

TABLE I.

Serologic Distinctness of Eastern, Western, and Venezuelan Equine Encephalomyelitis Viruses as Shown by Subcutaneous Cross Neutralization Tests in 3-Day-Old Mice.

Virus	Rabbit sera		Mortality ratio* of mice inoculated with serum plus virus dilution										Effective virus titer	LD_{50} doses of virus neutralized by serum
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}		
Eastern Equine Encephalomyelitis	Eastern E. E.	268	6/6	0/6	0/5	0/6	0/6						$10^{-1.5}$	50,000,000
	Normal	268					6/6	6/6	6/6	6/6	4/6	0/5	$10^{-9.2}$	
	Eastern E. E.	301	5/6	2/6	0/6	0/6	0/6						$10^{-1.6}$	50,000,000
	Normal	301						6/6	6/6	6/6	4/6	1/6	$10^{-9.3}$	
	Western E.E.	295					6/6	6/6	6/6	6/6	4/6		$10^{-9.2}$ or $>$	
	Normal	295						6/6	6/6	6/6	4/6	1/6	$10^{-9.3}$	0
	Western E. E.	298				6/6	6/6	6/6	6/6	6/6	4/6		$10^{-9.2}$ or $>$	
	Normal	298					6/6	6/6	6/6	6/6	4/6	0/6	$10^{-9.2}$	0
	Ven. E.E.	337				6/6	6/6	6/6	6/6	6/6	4/6		$10^{-9.2}$ or $>$	
	Normal	337					6/6	6/6	6/6	6/6	6/6	1/6	$10^{-9.6}$	2.5 or $<$
	Ven. E.E.	375					6/6	6/6	6/6	6/6	3/6		$10^{-9.0}$ or $>$	
	Normal	375						6/6	6/6	6/6	5/6	1/6	$10^{-9.5}$	3 or $<$
	French Neuro.†	306					6/6	6/6	6/6	6/6	4/6		$10^{-9.2}$ or $>$	
	Normal	306						6/6	6/6	6/6	4/6	0/6	$10^{-9.2}$	0
	French Neuro.	308					6/6	6/6	6/6	6/6	4/6	1/6	$10^{-9.3}$	
	Normal	308					6/6	6/6	6/6	6/6	6/6	0/6	$10^{-9.5}$	1.5 or $<$
Western Equine Encephalomyelitis	Western E. E.	295	2/6	0/6	0/6	0/6	0/6	0/5					$<10^{-1.0}$	$>100,000,000$
	Normal	295						6/6	6/6	6/6	3/6	0/6	$10^{-9.0}$	
	Western E. E.	298	1/6	0/6	0/6	0/6	0/6						$<10^{-1.0}$	$>$ 31,600,000
	Normal	298					6/6	6/6	6/6	6/6	0/6	0/6	$10^{-8.5}$	
	Ven. E. E.	337				6/6	6/6	6/6	6/6	4/6	2/6		$10^{-8.5}$	
	Normal	337					6/6	6/6	6/6	6/6	0/6	0/6	$10^{-8.5}$	0
	Ven. E. E.	375					6/6	6/6	6/6	5/6	1/6		$10^{-8.5}$	
	Normal	375						6/6	6/6	5/6	1/6	0/6	$10^{-8.5}$	0
	Eastern E. E.	268					6/6	6/6	6/6	6/6	0/5		$10^{-8.5}$	
	Normal	268					6/6	6/6	6/6	6/6	1/6	0/6	$10^{-8.6}$	0
	Eastern E. E.	301					6/6	6/6	6/6	2/6	3/6		$10^{-8.2}$	
	Normal	301						6/6	6/6	5/6	2/6	1/6	$10^{-8.7}$	3
	French Neuro.	306					6/6	6/6	6/6	6/6	3/6		$10^{-9.0}$ or $>$	
	Normal	306						6/6	6/6	6/6	3/6	0/6	$10^{-9.0}$	0
	French Neuro.	308					6/6	6/6	6/6	4/6	1/6	0/6	$10^{-8.3}$	
	Normal	308					6/6	6/6	6/6	2/6	2/6	0/6	$10^{-8.0}$	0

Venezuelan Equine Encephalomyelitis	Ven. E. E.	337	6/6	4/6	1/6	0/6	0/6	0/6					10-2.3		12,600,000
	Normal	337					6/6	6/6	6/6	6/6	5/6	0/6	10-9.4		
	Ven. E. E.	375	0/6	0/6	0/6	0/6	0/6	0/6					<10-1.0		> 31,600,000
	Normal	375						6/6	6/6	6/6	0/6	0/6	10-8.5		
	Eastern E. E.	268				6/6	6/6	6/6	6/6	6/6	1/6		10-8.6		
	Normal	268					6/6	6/6	6/6	6/6	0/6	0/6	10-8.5		0
	Eastern E. E.	301					6/6	6/6	6/6	6/6	4/6		10-9.2		
	Normal	301						6/6	6/6	6/6	2/6	1/6	10-8.8		0
	Western E. E.	295					6/6	6/6	6/6	6/6	6/6		10-9.5	or >	
	Normal	295						6/6	6/6	6/6	3/6	2/6	10-9.3		0
	Western E. E.	298				6/6	6/6	6/6	6/6	6/6	4/6		10-9.2		
	Normal	298					6/6	6/6	6/6	6/6	0/6	0/6	10-8.5		0
	French Neuro.	306					6/6	6/6	6/6	5/6	6/6		10-9.4	or >	
	Normal	306						6/6	6/6	6/6	6/6	0/6	10-9.5		0
	French Neuro.	308					6/6	6/6	6/6	6/6	2/6	0/6	10-8.7		
	Normal	308					6/6	6/6	6/6	5/6	1/6	0/6	10-8.5		0
Yellow Fever (French Neurotropic Strain)	French Neuro.	306	0/6	0/6	0/6	0/6	0/5						<10-1.0		>200,000,000
	Normal	306						6/6	6/6	4/6	1/6	0/6	10-9.3		
	French Neuro.	308	0/6	0/6	0/6	0/6							<10-1.0		> 10,000,000
	Normal	308					6/6	6/6	6/6	3/6	0/6	0/6	10-8.0		
	Ven. E. E.	337				6/6	6/6	6/6	6/6	6/6	1/6	0/6	10-8.6		
	Normal	337				6/6	6/6	6/6	6/6	5/6	2/6	0/6	10-8.6		0
	Ven. E. E.	375						6/6	6/6	5/6	0/5		10-8.4		
	Normal	375						6/6	6/6	3/6	0/6	0/5	10-8.0		0
	Eastern E. E.	268				6/6	6/6	6/6	6/6	5/6	1/6	0/6	10-8.5		
	Normal	268				6/6	6/6	6/6	6/6	6/6	1/6	0/6	10-8.6		0
	Eastern E. E.	301						6/6	5/6	3/6	1/6		10-8.0		
	Normal	301						6/6	6/6	4/6	0/6	0/6	10-8.2		1.5
	Western E. E.	295						6/6	6/6	4/6	0/6		10-8.2		
	Normal	295						6/6	6/6	4/6	3/6	0/6	10-8.7		3
	Western E. E.	298				6/6	6/6	6/6	6/6	5/6	6/6	0/6	10-9.4		
	Normal	298				6/6	6/6	6/6	6/6	4/6	0/6	0/5	10-8.3		0

* The mortality ratio is expressed by a fraction in which the numerator indicates the number of mice which died and the denominator the number of mice which were inoculated (less those destroyed by the mother of the litter).

† French Neuro. = French Neurotropic strain of yellow fever virus.

French neurotropic strain of yellow fever virus was included as a specificity control. Each experiment was subsequently repeated, using a different set of immune sera, to check the results of the first test.

Presentation of Results. The 50% mortality end point titer[13] of the virus in the presence of immune and of normal sera was calculated and is shown in the table under the heading "Effective virus titer"; the difference in the logarithms of the titer of the virus in the presence of an immune serum and of the corresponding normal serum is expressed arithmetically in the last column as the number of LD_{50} neutralized.

Results. The results of the cross-neutralization tests are presented in Table I. It is apparent that while the immune sera neutralized millions of LD_{50} of their homologous virus, their neutralizing effect on the heterologous viruses was nil—neutralization either was totally undemonstrable, or ranged from 1.5 to 3 LD_{50}, an amount of no significance since it falls within the range of experimental error of the method.

Sera which have a questionable, or definite but weak, neutralizing capacity by the intracerebral test usually, if not always, show a much greater neutralizing power by the subcutaneous or intraperitoneal test,[9,10] and if the 3 equine encephalomyelitis viruses possess any antigenic components in common, it would be expected that the extraneural test would reveal similarities in their antigenic constitution. The absence of cross-neutralization despite the high sensitivity of the test indicates, therefore, that the eastern, western, and Venezuelan equine encephalomyelitis viruses are serologically unrelated.

Why the results of cross-immunity tests should be at variance with those of cross-neutralization tests is not evident. The situation with the equine encephalomyelitis viruses is analogous to that which obtains with the West Nile-St. Louis-Japanese group, except that the reverse holds true—the viruses appear to be related by cross-neutralization tests,[14,15] but not by cross-immunity tests.[14,15] While it is possible that the existence of common antigens may be more readily demonstrable for one group of viruses by one method and for another group by a different method, the antithetic results of the immune reactions within a group stresses the need for further investigation of these reactions. With regard to the equine encephalomyelitis group, the consistent failure to demonstrate by neutralization tests any unequivocal relationship between these viruses points to the desirability of restudying the problem from the standpoint of the specificity of the cross-immunity tests, *i.e.*, has the cross-resistance demonstrated been due to an interference effect,[16] to the existence of paraimmunity,[17] or to an antibody response broadened by repeated antigenic stimuli, or is it due to an acquired specific cellular immunity whose breadth is not reflected by humoral antibodies.

Summary. The relationships suggested by cross-immunity tests to exist between the eastern, western, and Venezuelan equine encephalomyelitis viruses were investigated by means of cross-neutralization tests done in 3-day-old mice by the subcutaneous route. Despite the high sensitivity of the test, there was no evidence that these viruses possess any antigenic components in common.

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15109

Effects of Para-aminobenzoic Acid in Experimental Tsutsugamushi Disease (Scrub Typhus).

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Para-aminobenzoic acid (PABA) has been found to have a beneficial effect in experimental typhus[1,2] and in the treatment of classical louse-borne typhus fever in humans.[3] In the present report preliminary experiments are described which indicate that PABA may have a therapeutic effect against another rickettsial infection, experimental tsutsugamushi disease (scrub typhus).

The animals employed in these tests were *Gerbillus gerbillus* Olivier and *Gerbillus pyramidum* Geoffroy. Their susceptibility to tsutsugamushi disease has been described by Zarafonetis[4] who discussed the characteristics of the experimental infection produced by *R. orientalis* and cited the sources of the strains which were employed in the chemotherapy experiments presented below.

Four experiments are reported in this communication. In Experiments I and II, the "Imphal" strain was used. The inoculum was prepared from a moribund gerbille by washing the peritoneal cavity with 4 cc saline. One cc was injected intraperitoneally into young gerbilles (species *G. pyramidum*) which weighed 20 to 25 g. The control gerbilles were fed a diet consisting of dried, powdered Egyptian bread. For the treated group of gerbilles 3 g of powdered PABA were mixed with 97 g of dried bread. Water bottles were placed in the cages of both treated and control groups. The feeding of PABA was begun on the day of inoculation and was continued until all the animals were dead. In Experiment I no PABA was given parenterally. In experiment II one group of gerbilles received 10 mg of PABA subcutaneously twice daily, beginning on the 7th day after inoculation, approximately 36 hours before the average time of death of the control animals. Another group of gerbilles received no PABA either in the diet or subcutaneously until the seventh day. A few gerbilles were sacrificed for the measurement of blood concentrations of PABA; as expected, the blood levels fell to zero when the animals stopped eating with the onset of sickness.

The results of Experiment I and II are presented in Table I, which shows that there were no survivors in either experiment. There was a slight suggestion that the survival time was lengthened by the early addition of PABA to the diet. Subsequent tests showed that the inoculum probably contained between 100 and 1000 fatal doses.

In Experiments III and IV the "Ceylon" strain was used. The highly soluble sodium salt of PABA, referred to hereafter as NaPAB, was used instead of the relatively insoluble acid itself. The diet for the control gerbilles was a wet mash composed of 80 g of dried, powdered Egyptian bread, 2 to 4 g of sodium chloride, 60 cc of milk, and 40 cc of tap water. The diet for the treated gerbilles was identical except that 40 cc of 9% NaPAB were used instead of water in preparing the mash. Water bottles supplemented the diet of both treated and control groups. Feeding of the drug was begun immediately after inoculation and was continued until the experiments were arbitrarily terminated at the end of the 21st day. Since these rodents tend to eat only at night, the blood levels of PABA undoubtedly fluctuated widely, and it was decided to supplement the amount received in the diet by injec-

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1 Snyder, J. C., Maier, J., and Anderson, C. R., Report to the Division of Medical Sciences, National Research Council, Dec. 26, 1942.

2 Hamilton, H. L., Plotz, H., and Smadel, J. E., Report to the Director of The United States of America Typhus Commission, 16 December, 1943. To be published.

3 Yeomans, A., Snyder, J. C., Murray, E. S., Zarafonetis, C. J. D., and Ecke, R. S., *J. A. M. A.*, 1944, **126**, 349.

4 Zarafonetis, C. J. D., Proc. Soc. Exp. Biol. and Med., 1945, **59**, 113.

TABLE I.

Data from Experiments I and II with the "Imphal" Strain of Tsutsugamushi Disease. Each Gerbille Was Inoculated Intraperitoneally with 1 cc of Undiluted Peritoneal Washings (Inoculum "A").

Exp.	Description of groups	No. of gerbilles in each group	Survival time of each gerbille, days	Avg survival time of entire group, days
I.	Controls	6	9, 9, 11, 11, 12, 12	10.7
	PABA (3% in diet begun zero day)	5	9, 10, 11, 16, 16	12.4
II.	Controls	6	7, 8, 8, 8, 9, 10	8.3
	PABA (3% in diet begun zero day; s.c. from 7th day on)	6	8, 8, 8, 9, 11, 12	9.3
	PABA first begun 7th day in diet and s.c.	6	8, 8, 8, 8, 9, 10	8.5

TABLE II.

Data from Experiments III and IV, with the "Ceylon" Strain of Tsutsugamushi Disease.

Exp. No.	Concentrations of inoculum	Controls: Time of death of each gerbille, days after inoculation	Controls: Ratio survivors to total in group	NaPAB treated: Time of death of each gerbille days after inoculation	NaPAB treated: Ratio survivors to total in group
III.	"A." Undiluted	6, 9, 9, 13	0/4	14, *15*, 18	1/4
	"B." Diluted 1/10	12, 12, 13, 14	0/4	*8, 15*	2/4
	"C." " 1/100	11, 12, 13, 14	0/4	10*	3/4
	"D." " 1/1000	13, 18, 19	1/4	—	4/4
IV.	"A." Undiluted	9, 10, 10, 10	0/4	10, 12, *13*	1/4
	"B." Diluted 1/10	11, 11, 11	0/3	16	3/4
	"C." " 1/100	13, 13, 14, 15, 16	0/5	—	4/4
	"D." " 1/1000	20, 20, 20	1/4	—	3/3

* This gerbille was attacked by its cage mates on the 8th day, and a large area of skin was chewed away; it was isolated; at autopsy no exudate was found and the organs appeared to be normal; a very few rickettsiæ were seen in smears.

Where the day of death has been italicized, the autopsy findings were atypical, in that there were slight or no pleural and peritoneal exudates and the organs showed slight or no variation from normal in size and gross appearance. In 2 of these gerbilles, however, the liver was large and quite pale, a finding not previously encountered in tsutsugamushi disease in gerbilles.

TABLE III.

Summary of the Data from Experiments I, II, III, and IV Arranged to Show the Total Number of Gerbilles in the Control and the Treated Groups Which Received Different Concentrations of Infective Inoculum and the Percent Survival in Each Group.

Concentration of inoculum	Control gerbilles: Total No. in group	Control gerbilles: % survival	Treated gerbilles*: Total No. in group	Treated gerbilles*: % survival
"A" Undiluted	20	0	25	8
"B" Diluted 1/10	7	0	8	62½
"C" " 1/100	9	0	8	87½
"D" " 1/1000	8	25	7	100

* The dosage is described in the text.

tions of NaPAB at intervals of 8 hours. In Experiment III the parenteral administration was begun on the 7th day and continued until the end of the 16th day; the dose was 0.1 cc of 9% NaPAB. In Experiment IV the injections were started one day earlier and continued one day longer; the dose was increased to 0.15 cc of 9% NaPAB because the gerbilles were larger. In both experiments the controls were inoculated with 0.85% saline at the same intervals and in the same volumes as the treated gerbilles. No anesthesia was used. Titrations were made to determine the severity of the infecting dose of rickettsiæ. On 2 occa-

sions, blood PABA concentrations were measured 8 hours after a subcutaneous dose of 9 mg of NaPAB. The values were 3 and 5 mg per 100 cc, which were taken to indicate that the parenteral dosage schedule as supplemented by the amount taken in the diet probably was adequate to maintain a measurable concentration of PABA in the blood throughout the period of therapy.

In Experiment III most of the gerbilles were of the species *G. pyramidum,* though a few were *G. gerbillus*. The animals weighed less than 30 g at the beginning of the test. The inoculum consisted of peritoneal washings only. Table II shows that of a total of 16 gerbilles in each category, only 1 control gerbille survived, whereas 10 NaPAB treated gerbilles survived.

In Experiment IV all of the gerbilles were of the species *G. pyramidum*. The weight range was 30 to 40 g at the outset. The inoculum consisted of peritoneal washings in which brain and spleen of the same gerbille were suspended, so that the concentration of these organs in the "undiluted" inoculum was approximately 10%. It can be seen in Table II that only 1 control gerbille survived out of a total of 16 inoculated, whereas 11 of 15 NaPAB treated gerbilles survived.

The findings at autopsy in all of the control gerbilles were characteristic of experimental tsutsugamushi disease as previously reported, and rickettsiæ were demonstrated in smears of the peritoneal exudates. In the NaPAB treated groups, however, atypical findings were encountered as shown in the footnotes of Table II.

A summary of the 4 experiments has been prepared in Table III which shows the total number of animals and the percent survival in each group.

It seems clear from the data shown in Table III that the inoculum consisting of undiluted peritoneal washings (inoculum "A"), which contained roughly 100 to 1000 fatal doses, was so severe that the effect of PABA, or NaPAB, was overwhelmed; there were no survivors in the control groups (20 gerbilles) and only 2 survivors in the treated groups (25 gerbilles).

With inoculum "B," which contained roughly 10 to 100 fatal doses a definite difference was apparent. There were no survivors in the controls, as contrasted with 5 of 8 or 62½% in the NaPAB treated groups.

With inoculum "C," which contained between 1 and 10 fatal doses, the difference was even greater: none of the control gerbilles survived, as opposed to 7 of 8 or 87½% of the NaPAB treated gerbilles.

Inoculum "D," was not sufficiently concentrated to kill all of the control animals within the arbitrary 21-day limit. Two control gerbilles survived of a total of 8 inoculated. There were no deaths in the treated groups, however.

These results must be confirmed by tests using larger numbers of animals and other strains of *R. orientalis* before final conclusions can be drawn. The evidence thus far is highly suggestive, however, that the mortality from experimental tsutsugamushi disease can be reduced by the administration of the sodium salt of para-aminobenzoic acid.

Summary. The oral and parenteral administration of the sodium salt of para-aminobenzoic acid to gerbilles (*Gerbillus gerbillus* and *Gerbillus pyramidum*) experimentally infected with *R. orientalis* reduced the mortality. The results of these preliminary experiments indicate that this compound may have beneficial therapeutic action in the treatment of tsutsugamushi (scrub typhus) infection.

The assistance of Corporals Stearman, Hogan, Cassel, and Friedberg is gratefully acknowledged.

15110

Experimental Hyperplasia of the Stomach Mucosa.

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The mucosa of the human stomach presents striking individual differences in area and in thickness.[1] These are often unrelated to any distinct qualitative differences in mucosal structure. It has been noted in further studies in this laboratory that the thickness of the mucosa of the specific secretory portion of the human stomach is particularly subject to marked individual difference, and that there is a roughly parallel diversity in the number of parietal, or acid secreting, cells. This variation has been seen to occur in stomachs which would otherwise be classified as normal, and it has suggested the possibility that stomachs with large numbers of secreting cells may be instances of hyperplasia associated with increased functional activity.

Because of numerous demonstrations of increases in number and size of structural elements of many different organs in association with increased function, it should be expected that gastric mucosa might undergo hyperplasia, if its secretory activity is increased. The following experiment was designed to induce this state in guinea pigs by prolonged stimulation with injected histamine according to the method of Code and Varco.[2]

Methods. Fourteen young guinea pigs of both sexes, ranging in weight from 310 to 830 g, were injected intramuscularly with 3 to 6 mg of histamine phosphate* in beeswax-mineral oil mixture 3 times weekly for from 2 to 4 weeks. The animals were sacrificed by bleeding while under ether anesthesia. The stomachs were removed immediately, opened along the greater curvature, stretched on cardboard to remove all mucosal folds, and then were fixed in 4% formaldehyde solution. A map of each fixed specimen was prepared by tracing the outline upon paper, and the location of 3 excised strips of gastric wall was plotted upon it. These strips were representative samples of the dorsal and ventral walls and lesser curvature. Longitudinal histological sections 8 microns thick were stained with Giemsa stain, and the amount of linear shrinkage of the strips during the preparation was determined. The junction of the acid secreting portion of the mucosa with that of the pyloric zone was identified in the sections, and after an appropriate correction, was plotted upon the map. A line joining the 3 points thus obtained, determined the approximate position of the junction between the 2 mucosal zones. The area of the acid secreting zone was measured in 3 parts with a planimeter: the ventral wall, the dorsal wall, and the lesser curvature. The latter was defined arbitrarily as the zone within lines joining the

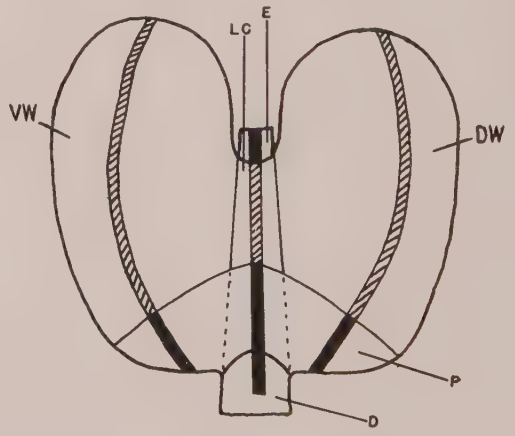

Fig. 1.

Diagram of an opened, stretched guinea pig stomach showing the 3 excised strips and the mucosal zones. The striped portions of the strips show the location of parietal cells. D = duodenum, E = esophagus, P = pyloric zone, VW = ventral wall, DW = dorsal wall, LC = lesser curvature.

1 Cox, A. J., *Calif. and Western Med.*, in press.

2 Code, C. F., and Varco, R. L., *Am. J. Physiol.*, 1942, **137**, 225.

* The histamine phosphate (Ergamine phosphate) was supplied in part through the courtesy of Burroughs Wellcome & Co.

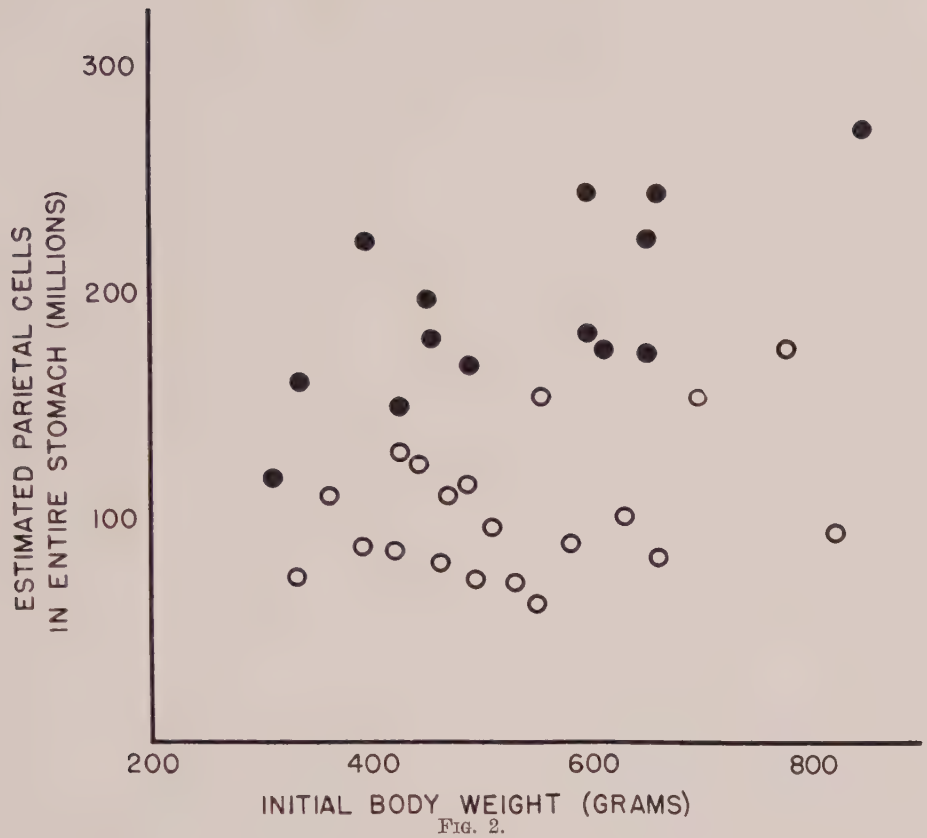

FIG. 2.

Estimated total number of parietal cells in guinea pig stomachs after prolonged administration of histamine. ● = treated animals; ○ = control animals.

edges of the opened duodenum with those of the opened esophagus (Fig. 1).

In each stomach, from the histological sections which were cut perpendicular to the mucosal surface, 36 counts were made of the number of parietal cells in representative bands 0.23 mm broad extending across the sections from the surface to the base of the mucosa. Only cells in which nuclei were visible were counted; this eliminated the necessity of identifying cells which were barely included in the section, and permitted the estimation of an "effective thickness" of the section by adding the average measured diameter of the parietal cell nuclei to the actual thickness of the section. This average nuclear diameter (5 microns) was the same in treated and control animals. From the representative counts and the measured areas, an estimated total count was calculated for each of the 3 regions mentioned above, after correcting for shrinkage in length of the specimen during preparation. By addition, an estimated total number of parietal cells for each entire stomach was obtained. No correction was made for shrinkage in width of the prepared strips of stomach wall, but this could not be determined accurately, and since shrinkage in length was essentially the same in the treated and untreated animals, it was assumed that shrinkage in width would also be similar in the two groups.

Results. The estimated numbers of parietal cells in the different stomachs are shown in Fig. 2. The controls were 20 animals of similar weight and sex, and in some instances were littermates of the treated animals. It can be seen that in the stomachs of the treated group, where it can be assumed that there had been prolonged increased secretion of acid,[2,3]

[3] Code, C. F., and Varco, R. L., PROC. SOC. EXP. BIOL. AND MED., 1940, **44**, 475.

there were distinctly more parietal cells. No difference could be attributed to the sex of the animals, and the size of the injected quantities of histamine was not clearly related to the degree of response. However, the animals which were subjected to the longer series of injections showed somewhat greater increases in parietal cells than did those which were injected for only 2 weeks. Five additional similarly treated animals died with perforated pyloric or duodenal ulcers before completion of the injections. No animals with ulcers were used for estimation of parietal cell increases.

Estimation of the number of other cell types in the mucosa is more difficult than reckoning the parietal cells, which are usually clearly outlined and easily identified. Therefore, counts of other cell types have not been made.

Nothing was seen to suggest that the increase in number of parietal cells was due to any process other than true hyperplasia. No partially granulated parietal cells were recognized. It is probable that the increase in cells represents a response to stimulation of function.

Summary and Conclusion. An increase in number of parietal cells occurs in the mucosa of the guinea pig stomach after protracted stimulation with histamine over a period of 2 to 4 weeks. This is presumably a hyperplasia and may indicate a mechanism to explain differences in the number of secreting cells in different human stomachs.

15111

Effect of Digitalis on Cholinesterase.

OVIDIO MIQUEL AND WALTER F. RIKER, JR. (Introduced by McKeen Cattell.)

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The mechanism through which digitalis brings about a slowing in cardiac rate has been the subject of a number of views. Abdon *et al.*[1] have suggested that the digitalis bradycardia might be explained as the result of a sensitization of the myocardium to acetylcholine, possibly through the inhibition of cholinesterase. These workers repeated certain experiments of Matthes[2] using a colorimetric method for the determination of acetylcholine, and were in agreement with him in finding no inhibition of the enzymatic breakdown of acetylcholine by *g*-strophanthin in concentrations up to 1:5000.

No data other than these summary statements of a negative result are given in these reports, and other references to the effect of digitalis on cholinesterase have not been found. Further evidence on this point has been obtained with a specific cholinesterase prepared from the electric organ of the eel as well as with non-specific human serum esterase.

The following digitalis glycosides were studied: digitoxin, ouabain, strophanthin, and lanatoside C. The effect of each of these substances was tested on both serum esterase and eel cholinesterase, using a concentration range approximating that which might obtain in the blood following a therapeutic dose, as well as higher concentrations. For the determination of cholinesterase activity the Warburg manometric technic was employed. Dilution of the glycosides was made in saline, and 0.5 cc of this was added to 3 cc of a 0.02 M solution of acetylcholine in the vessel. The acetylcholine solution was made by dissolving 225.9 mg of acetylcholine bromide in 50 cc of 0.03 M sodium bicarbonate. In the case of controls, saline alone in an equivalent amount was added to the flask. The enzyme dilution (0.5 cc) was placed in the side bulb and later tipped into the reaction mixture. The final dilution of human serum after tipping was 1:40, and of the eel esterase 1:3200.

[1] Abdon, N. O., and Nielsen, N. A., *Skand. Arch. Physiol.*, 1938, **78**, 13.

[2] Matthes, K., *J. Physiol.*, 1930, **70**, 338.

TABLE I.

Glycoside	Dilution, × 1000	Esterase	mm^3 CO_2/30 min Control	with glycoside	% change
Digitoxin	1:5,000	Eel	130.69 (3)*	132.14 (3)*	+ 1.1
"	1:1,000	"	134.60 (3)	152.64 (3)	+13.4
"	1:1,000	Serum	165.59 (3)	178.64 (3)	+ 7.9
"	1:100	Eel	109.92 (3)	124.20 (3)	+13.0
"	1:10	Serum	196.55 (3)	191.08 (3)	— 2.8
Ouabain	1:250	Eel	73.00 (1)	83.40 (1)	+14.2
"	1:250	Serum	142.80 (1)	150.48 (1)	+ 5.4
"	1:100	Eel	91.44 (3)	88.85 (3)	— 2.8
"	1:10	"	73.17 (3)	81.64 (3)	+11.6
"	1:10	Serum	188.64 (3)	173.79 (3)	— 7.9
Strophanthin	1:500	Eel	98.59 (2)	85.54 (3)	—13.2
"	1:500	Serum	181.06 (3)	189.04 (3)	+ 4.4
"	1:10	"	186.72 (3)	208.99 (3)	+11.9
Lanatoside C.	1:40	Eel	73.70 (2)	81.10 (3)	+10.0
" "	1:40	Serum	220.74 (3)	221.87 (3)	+ 0.5

* The numbers in parentheses indicate the number of determinations and the value shown is the average.

The flasks were equilibrated with a mixture of 5% carbon dioxide and 95% nitrogen, and allowed to reach thermal equilibrium in the bath at 37°C. After an initial reading the vessels were tipped and readings made at 10-minute intervals for a period of one to 2 hours. Controls were run simultaneously in each experiment.

Using the straight line portion of the hydrolysis curve over a period of one-half hour, the results were expressed as the number of mm^3 of carbon dioxide liberated per 30 minutes.

Table I shows the results of 15 experiments on the effect of the different dilutions of the various glycosides on both serum and eel esterase. In general there is no significant alteration in the activity of either enzyme as a result of the addition of digitalis. An average increase for all the glycosides and all dilutions of 3.1% for eel esterase and 2% for the serum esterase took place but in view of the individual variations these differences cannot be regarded as significant.

The serum esterase activity of 2 cats was determined at a time when the animals were near death as a result of intravenous doses of digitoxin, given at 5-minute intervals. One cat received 0.444 mg/kg over a period of 97 minutes, and the other 0.675 mg/kg of digitoxin in 135 minutes. The esterase activity of the serums taken just prior to death in each case was not significantly different from the values obtained before digitalis.

Summary. These results confirm the observation that the cardiac slowing by digitalis is not due to inhibition of cholinesterase.

15112

Exaggerated Discharge of Neurosomes into Spastic Muscle by Heat Reflex.*

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Morphologic evidence of the transmission of nervous substance into the myoplasm of spastic muscle from the motor end plates has not been demonstrated. Neurosomes were first described as "auriphilic inclusion masses"

* Aided by a grant from The National Foundation for Infantile Paralysis, Inc., and the Baruch Committee on Physical Medicine.

in the abnormal muscles of the monkey[1] and man[2] during the early stages of poliomyelitis; after injection of lactic acid[3] into rat's muscle; and during the onset of chemical,[4] electric,[5] hemorrhagic,[6] traumatic,[7] and thermal[8] shock. The purpose of this study is the morphologic demonstration of neurosomes discharged from the large motor end plates into the muscles of chameleons immediately after their spastic or cramp-like response to the reflex action of heat applied to the skin.

Methods. The following easily reproducible experiments were made. Ten chameleons (*Anolis caroliniensis*) 3 to 5 g were decapitated. The 20 biceps femoris muscles were excised and used as controls after they were subjected to various histologic technics, viz., gold,[5] Bielschowsky silver, methylene blue, osmic acid, sudan III, Herxheimer's alkaline scarlet red in both teased and sectioned tissue. The freshly teased muscles, frozen longitudinal and cross sections were observed.

Additional control experiments were also performed by the following method. The spinal cord was destroyed from the base of the tail to a level 2 cm cephalad by the pithing method with a teasing needle in 40 unanesthetized chameleons. Both hind extremities were in a state of flaccid paralysis after pithing the cord. There was no reflex response of any hind limb upon amputation of a toe with a pair of scissors. The caudal one-half of each animal was then immersed in water 70°C for one second after the following time intervals subsequent to pithing the spinal cord: 6 animals immersed immediately, 8 animals immersed after 2 hours, and 26 animals after 8 hours. There was no reflex response of the muscles of the hind limbs to the thermal stimulus applied to the skin. The tails of the immersed animals with the intact caudal part of the spinal cord, however, manifested reflex responses by rapid lateral and circular movements. The 80 hind limbs were skinned and excised immediately after immersion of the animals. The biceps femoris muscles were then subjected to the gold technic.

Fifty living unanesthetized and unskinned chameleons were vertically immersed with the hind limbs, in an extended position, in water 70°C over the caudal one-half of their bodies for 1 second. Forty chameleons had immediate extensor spastic paralysis of both hind legs; 10 were not visibly paralyzed. The 50 animals were divided into the 4 following groups: (1) 10 of the spastic animals were decapitated immediately; (2) 10 spastics were decapitated after 2 hours; (3) 10 spastics and the 10 non-spastics were decapitated after 4 hours; (4) 10 spastic animals were allowed to live 24 hours at which time 8 had fully recovered, 2 still had rigidity of both hind limbs; these 10 animals were decapitated. The 100 bilateral biceps femoris muscles were excised from the 50 experimental animals and subjected to the same histologic methods as the 20 muscles from the 10 normal controls.

Results. Both hind limbs of 40 of the 50 chameleons immersed in water 70°C, 1 second, immediately had spastic paralysis. This was dominantly a reversible rigor or reflex cramp of the muscles because 8 of 10 animals fully recovered after 6 to 24 hours. The green color of the immersed part of the skin became dark brown.

The relatively normal motor end plates in the 10 decapitated control chameleons had a mean for the length of 1000 end plates of 87.6 μ and for the width of 32.9 μ. Some of the end plates were retracted (Fig. 1) with lack of definition between the terminal axons and the granules of Kühne. They were frequently associated with few coarse cross striations. The expanded end plates (Fig. 3) had a decrease in the granules of Kühne and were associated with many fine cross striations.

1 Carey, E. J., PROC. SOC. EXP. BIOL. AND MED., 1943, **53**, 3; *Am. J. Path.*, 1944, **20**, 961.

2 Carey, E. J., Massopust, L. C., Zeit, W., and Haushalter, E., *J. Neuropath. and Exp. Neurol.*, 1944, **3**, 121.

3 Carey, E. J., and Massopust, L. C., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 194.

4 Carey, E. J., *Am. J. Path.*, 1944, **22**, 341.

5 Carey, E. J., *Am. J. Path.*, 1942, **18**, 237.

6 Carey, E. J., Massopust, L. C., Zeit, W., Haushalter, E., and Schmitz, J., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 115.

7 Carey, E. J., Massopust, L. C., Zeit, W., and Haushalter, E., *J. Neuropath. and Exp. Neurol.*, 1945, **4**, 134.

8 Carey, E. J., Massopust, L. C., Zeit, W., and Haushalter, E., *Am. J. Path.*, in press.

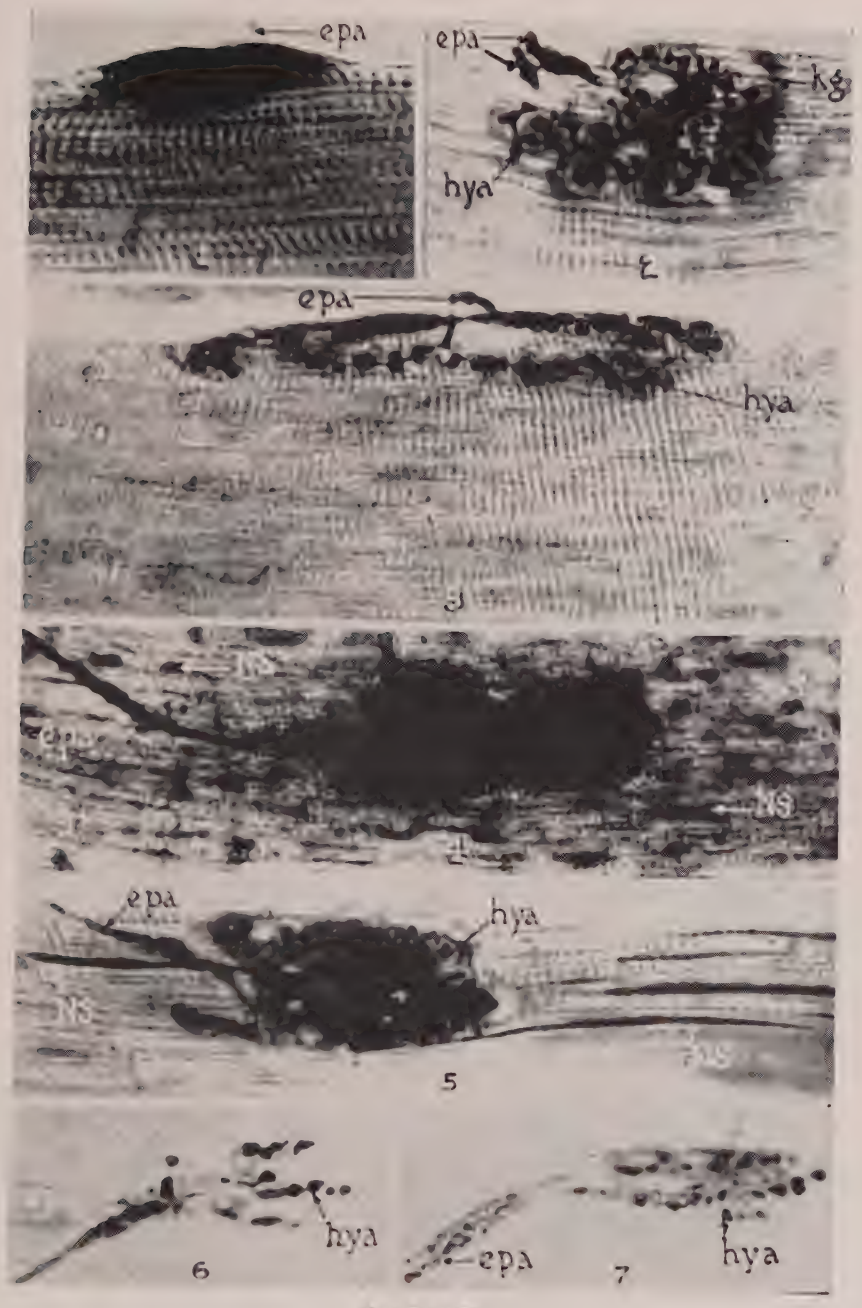

FIG. 1 TO 7.

Photomicrographs, normal innervation, Fig. 1 to 3, biceps femoris muscle, chameleon, and after thermal trauma, Fig. 4 to 7; epa, epilammal axons; hya, hypolemmal axons; Kg., Kühne's granules; NS, neurosomes; gold chloride, teased whole muscle fibers. × 500.

Intermediate stages were found (Fig. 2) in which both the terminal axons and the granular sole of Kühne were clearly defined. This granular rim varied from 1 to 30 μ in width and either gradually or suddenly blended with the dark anisotropic bands of the cross striations. These cross striations were absent around some end plates. They were replaced

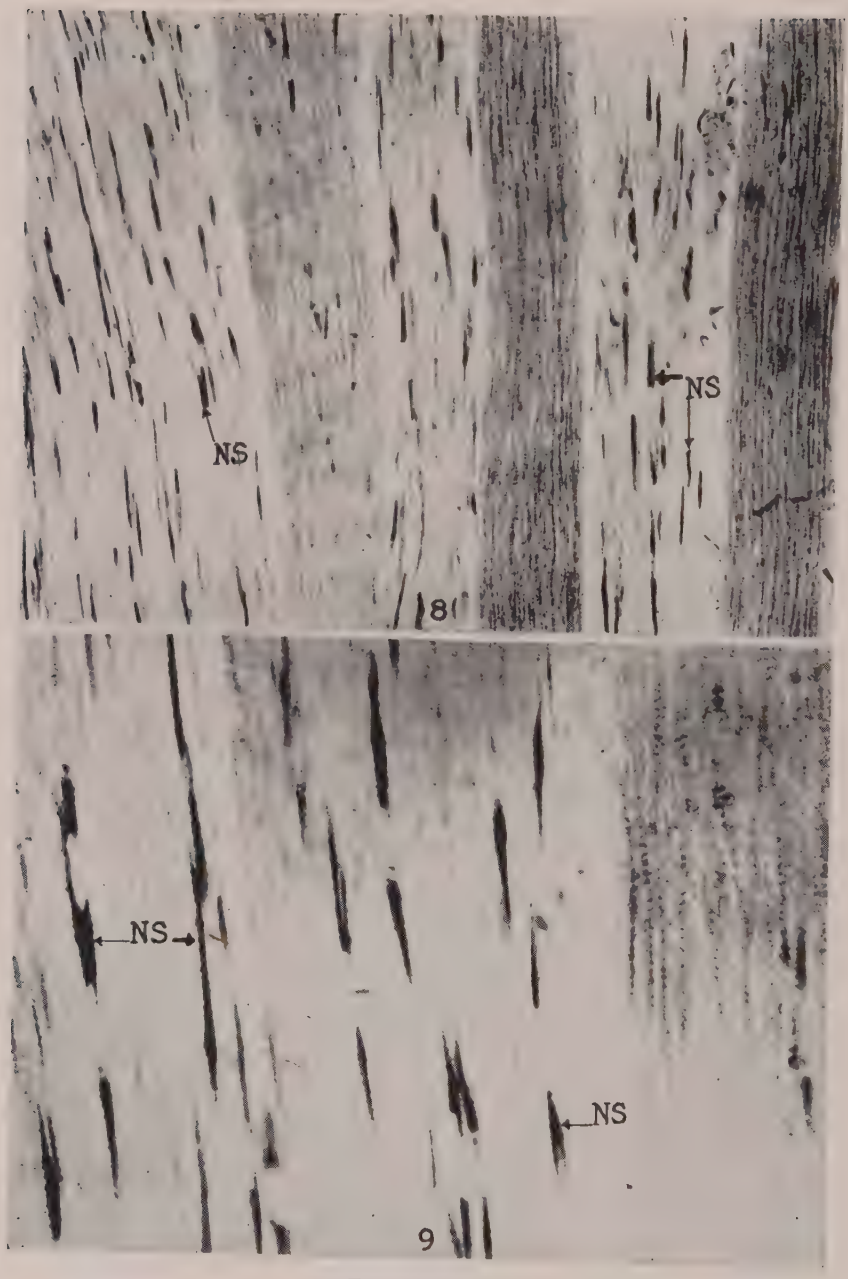

FIG. 8 AND 9.

Photomicrographs, biceps femoris muscle, chameleon, 2 hours after thermal trauma, 1 sec. duration of the skin. Neurosomes (NS.) dispersed in many muscle fibers; gold chloride, teased whole muscle fibers; Fig. 8, × 200; Fig. 9, × 500.

by a diffuse arrangement of granules. The retracted end plates (Fig. 1) had an increased capacity for gold; the expanded ones (Fig. 3) a decreased capacity.

The sequence of the morphologic effects of the reflex action of heat applied to the skin upon the motor end plates were the following: (1) increased capacity of the end plates for gold (Fig. 4, hyperchrysophilia) to the point of complete lack of definition between the

terminal axons and their transformation into the granules of Kühne; (2) exaggerated discharge of neurosomes or gold-impregnated bodies from the motor end plates into the myoplasm of the skeletal muscle fibers (Fig. 4 and 5, NS., neurosomes; axonorrhea); (3) progressive depletion to the level of complete structural exhaustion of the terminal axons of their substances with an affinity for gold (Fig. 6 and 7, hypochrysophilia; achrysophilia).

The neurosomes were found in 1 of the 20 relatively normal control muscles. They were present in a variable number of muscle fibers in 29 (Fig. 4 and 5, 8 and 9 NS.) of the 40 muscles of the experimental animals in Groups 1 and 2. Some of the muscle fibers had as high as 25% of their content composed of neurosomes intensely impregnated with gold. Some of the neurosomes were opaque, others were cross striated and clearly defined from the lightly impregnated cross striations of the muscle fiber. Neurosomes were found in variable amounts in 7 of the 20 spastic muscles and 3 of the 20 non-spastic muscles of the animals in Group 3. They were likewise distributed in a localized manner in 2 of the 16 fully recovered muscles and in 1 of the 4 spastic muscles of Group 4. These neurosomes, therefore, were greatly increased in frequency over that of the normal during the first 2 hours after heat trauma applied to the skin. Their disappearance appeared to be a function of the time interval after trauma and recovery of the animals from the reflex muscle rigor.

Neurosomes were found in scattered muscle fibers in 3 of the 12 muscles excised immediately after pithing of the spinal cord and immersion of the animals. The muscles of the hind legs underwent vigorous contraction during destruction of the spinal cord. Neurosomes were present in local areas in 1 of the 16 paralyzed muscles excised 2 hours after pithing of the spinal cord, at which time the animals were immersed. Neurosomes were also found in 1 of the 52 paralyzed biceps femoris muscles excised 8 hours after destruction of the spinal cord. These neurosomes were, therefore, slightly increased in frequency over that of the normal number in muscles excised immediately after pithing of the spinal cord and immersion of the animal. This increase of neurosomes was probably incident to the augmented neuromuscular activity associated with the traumatic destruction of the spinal cord. The neurosomes, however, were greatly decreased in frequency, under that of the normal number, in the paralyzed biceps femoris muscles excised 8 hours after destruction of the spinal cord. The great reduction in the number of neurosomes after spinal cord destruction was especially striking in comparison to that found in the spastic muscles of immersed animals with their spinal cords intact.

Neurosomes varied in morphology from the normal fine mantle composed of granules 0.5 to 2 μ in diameter of the sole of Kühne (Fig. 2, Kg) to oblong and fusiform bodies 10 to 1000 μ in length (Fig. 4, 5, 8, and 9, NS). There were droplets and vacuoles 5 to 35 μ in diameter (Fig. 5) in direct anatomic continuity with the terminal axons of some end plates. Some neurosomes were shaped like arrow heads and others had elongated irregular oblong forms. These dense neurosomes quickly underwent granulation, the granules of which became aligned into cross striations. During the first 2 hours after immersion some of the greatly elongated streamers resembled the ultraterminal non-medullated branches projected from the axons of the end plates observed infrequently in some control animals after decapitation. The ends of these ultraterminal branches varied in shape, viz., enlarged knobs, vacuoles, or gradually became attenuated into invisibility by incorporation into the myoplasm.

The neurosomes were inconstant in their capacity for gold, silver, osmic acid, sudan III, and scarlet red. With lipoidal stains the liposomes were spherical, oval, or fusiform in shape and varied in size and staining capacity. They had the appearance of non-nucleated fat droplets. They varied in refractile properties. Some large neurosomes had a myelin figure of a central maltese cross when observed with polarized light. When they were initially discharged during the first 2 hours after reflex action of thermal trauma applied to the skin, they had increased capacity for the above indicators. After the neurosomes had become incorporated into the myoplasm, they were

found for a time (4 to 24 hours after immersion) in some muscle fibers as fine, transversely aligned granules but in others they had completely disappeared.

Many of the muscle fibers had an increase of thick, dark, longitudinal myofibrils and a decreased prominence of the cross striations. In others, the cross and longitudinal striations completely disappeared and were replaced by granules or hyaline masses comparable to Zenker's waxy changes in muscle as well as abnormal waves of contraction. These were reversible waves because they were absent after recovery. The nuclei were frequently pycnotic especially in those fibers with an abundance of neurosomes. The pathologic changes in the myoplasm appeared to be in direct proportion to the number of neurosomes present during the first 2 hours after immersion of the animal. From 4 to 24 hours after immersion, in the 4 muscles of the 2 chameleons that remained spastic, there was progressive depletion of the end plates (Fig. 6 and 7), dissolution of neurosomes, and a more extensive pathologic change of the muscle than during the first 2 hours. During this late period after immersion, the muscular capillaries in many places were dilated, contained clumps of red blood cells, and had perivascular edema.

Discussion. The term *neurosomes* is herein defined as ephemeral, pleomorphic, and lipoidal bodies discharged into the myoplasm of striped muscle from superpermeable terminal axons of motor nerve plates: these neurosomes are composed of granules, droplets, vacuoles, or elongated streamers that vary in size and staining capacity for gold, silver, and lipoidal stains.

The current confusion regarding the true structure of the neuro-myoplasmic continuum composed of inconstant myofibrillæ, cross striations, sarcoplasmic granules, and the pleomorphic neuromuscular apparatus has been emphasized by Cobb[9] and Hinsey[10], respectively, and by Jordan,[11] Hines,[12] Tower,[13] Denny-Brown,[14] Boeke,[15] Wilkinson,[16] Murray,[17] and many others.

The relationship or identity of neurosomes with the interstitial granules of Kölliker[18] and the liposomes of Albrecht[19] is now under investigation. The experimental, tinctorial, and chemical methods used by Bell[20] to increase, decrease, and identify liposomes in muscle are precisely those that accentuate, eliminate, and identify the discharge of neurosomes from the motor end plates. In the past, the study of liposomes in muscle has not been correlated with the structural and staining changes of the motor end plates and those in striped muscle. When the histologist has supplied the needed data of the structural variations of the neuromuscular apparatus the pathologist will have criteria with which evaluations may be made of early changes of morphology involved in the onset of shock due to multiple extrinsic and intrinsic factors as well as of the so-called fatty metamorphosis of muscle. Evidence now at hand supports the above statements in their application to cardiac as well as skeletal muscle.

Summary. The limited experimental evidence presented tends to support the statement that the reflex effects of heat applied to the skin are associated with the exaggerated discharge of ephemeral, pleomorphic, and lipoidal neurosomes from the motor end plates into spastic or cramp-like muscle. The neurosomes have a variable affinity for gold, silver, and lipoidal stains. There are characteristic instantaneous changes in the morphology of

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the motor end plates and myoplasm in response to the reflex effects of thermal trauma applied to the skin.

Grateful ackowledgment is expressed for technical assistance to: Miss Estelle Downer; James Raggio, Silvio Davito, Chris Saribalis, James Sweeney, and Eli Socolof; to the 1944 and 1945 freshman classes for laborious aid in the teasing of muscle.

15113 P

Studies on Mode of Action of Streptomycin. I. Effect of Culture Media.

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(Introduced by F. W. Tanner.)

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In studying the mode of action of penicillin on bacteria, Hobby, Meyer, and Chaffe[1] and Lee, Foley, and Epstein[2] observed that multiplication must take place to get killing action and that the more rapid the growth of bacteria the greater the rate of destruction. Waksman and Woodruff[3] found the reverse to be true in the case of actinomycin and streptothricin. With these two antibiotics it was more difficult to inhibit bacteria on a good medium than it was on a poorer medium. The study reported here is an attempt to determine the action of streptomycin on bacteria and to see if this action was similar to either the action of penicillin or of actinomycin and streptothricin.

The streptomycin was prepared at the University of Illinois in the laboratories of H. W. Anderson and purified and standardized in the laboratories of H. E. Carter.[4,5] The unit used is practically the same as the Waksman dilution unit.[6]

Eberthella typhosa (Hopkins strain) and *Staphylococcus aureus* (FDA 209) were used as the test organisms, and were tested against the streptomycin according to the following procedure.

The following media were compared: (1) nutrient broth, (2) nutrient broth diluted with an equal amount of water, and (3) brain heart infusion. Because the presence of phosphate and NaCl might affect the action of streptomycin it was thought not advisable to use Difco brain heart infusion, but instead the brain heart infusion was made according to the following procedure. Five hundred g of ground beef heart were infused with 1000 ml of water and 500 g of beef brain were similarly treated. These infusions were separately filtered through gauze, heated to boiling and filtered again. The medium was made from these infusions using the following formula:

Beef heart infusion	75 ml
Beef brain infusion	25 "
Peptone (Bacto)	1 g
Glucose	0.2 "

Adjusted to pH 7.0 and sterilized.

Streptomycin was added in varying amounts to tubes containing 15 ml of each of these media and *Eberthella typhosa* and *Staphylococcus aureus* were added. Twenty-four-hour broth cultures of the organism were diluted with twice their volume of sterile water in order to obtain an inoculum which would not be too heavy and 0.1 ml of this suspension was used as the inoculum. Immediately thereafter a bacterial plate count was made on each tube

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3 Waksman, S. A., and Woodruff, H. B., *J. Bact.*, 1942, **44**, 373.

4 Carter, H. E., Clark, R. K., Jr., Dickman, S. R., Loo, Y. H., Skell, P. S., and Strong, W. A., *J. Biol. Chem.*, in press.

5 Loo, Y. H., Skell, P. S., Thornberry, H. H., Ehrlich, John, McGuire, J. M., Savage, G. M., and Sylvester, J. C., *J. Bact.*, in press.

6 Waksman, S. A., *Science*, 1945, **102**, 40.

TABLE I.

Action of Streptomycin on *Eberthella typhosa* and *Staphylococcus aureus* in Dilute Nutrient Broth, Nutrient Broth and Brain Heart Infusion.

	Viable organisms per ml in ten thousands								
	Eberthella typhosa					*Staphylococcus aureus*			
Medium	Amt streptomycin added in units	Hrs after inoculation 0	3	6	9	Amt streptomycin added in units	Hrs after inoculation 0	3	6	9
½ NB	0	20	70	*	*	0	40	40	50.	100.
,,	4.0	50	10	1.0	.5	7.5	10	6	.06	.03
,,	4.5	30	5	0.8	.3	8.0	20	7	.07	.02
,,	5.0	40	2	0.5	.3	8.5	20	7	.04	.01
NB	0	80	260	*	*	0	30	40	80.	140.
,,	4.0	40	40	4.	3.	7.5	10	4	2.	1.5
,,	4.5	40	30	6.	5.	8.0	50	4	1.	.6
,,	5.0	40	5	5.	3.	8.5	30	4	.5	.1
BHI	0	40	210	*	*	0	5	20	150.	300.
,,	4.0	60	190	*	*	7.5	15	7	3.	150.
,,	4.5	50	170	*	*	8.0	30	1.5	.7	620.
,,	5.0	40	230	*	*	8.5	20	1.3	.5	540.

NB—Nutrient Broth.
½ NB—Dilute Nutrient Broth.
BHI—Brain Heart Infusion.
* Too numerous to count.

to determine the density of the inoculum and at 3, 6, and 9 hours counts were repeated to determine the action of the streptomycin. In the first few series an attempt was made to determine the bacterial numbers nephelometrically with a Model 400 Lumetron (photoelectric colorimeter). Since the plate counts were found to be much more reliable, however, the nephelometric method was discontinued. All incubation was done at 37°C. The amounts of streptomycin found to be effective were 4.0, 4.5, and 5.0 units for *Eberthella typhosa* and 7.5, 8.0, and 8.5 units for *Staphylococcus aureus*. The streptomycin was prepared so that one unit was contained in 0.1 ml of sterile water.

Table I summarizes the results of the tests. They were repeated several times and the results checked on all occasions.

The table indicates that the action of streptomycin is not like that of penicillin but more like that of actinomycin and streptothricin as observed by Waksman and Woodruff. This was especially true with respect to cultures of *Eberthella typhosa* which showed active growth in brain heart infusion while those in nutrient broth and dilute nutrient broth rapidly decreased in numbers. There seemed to be a greater decrease in the diluted nutrient broth than in the full strength nutrient broth. The same held true for the *Staphylococcus aureus*, but the growth was slower and not as abundant in the brain heart infusion as it was in the case of *Eberthella*. In fact there was a decrease at the third and sixth hours which indicated killing action and then a marked increase. It is possible that this may mean that the streptomycin is used up in the action. This is being studied.

It is apparent that there is something present in the brain heart infusion which interferes in some way with the action of streptomycin which is not present in the nutrient broth.

15114

Studies on Antidromic Vasodilatation. II. Synaptic Connections in the Dorsal Root Ganglion.*†

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Neuroanatomy and histology textbooks discuss, if only briefly, those synapses described by Dogiel which are to be found in the dorsal root ganglion. However, the further connections of these synapses are, as yet, obscure.

Hasama[1] painted .2% nicotine on the dorsal root ganglia in the toad and was then unable to obtain the vasodilatation previously elicited by stimulation of the peripheral stump of these roots sectioned between the ganglion and the cord. An intravenous injection of 1% nicotine tartrate was found by Kuré *et al.* to prevent vasodilatation produced by the stimulation of the peripheral stumps of the lumbar and sacral dorsal roots sectioned between the ganglion and the cord in dogs.[2] These workers concluded that para-sympathetic efferent fibers synapse in the dorsal root ganglia. Ranson[3] and Ranson and Wightman[4] have shown that reflexly induced vasodilatation is definitely prevented by intravenous administration of nicotine in dogs and that the reflex pathway concerned in this response does synapse in the dorsal root ganglia. Sheehan,[5] in a recent review, expresses the opinion that the preponderance of evidence indicates that dorsal root synapses do occur.

Wybauw[6] found that a 1% solution of nicotine tartrate injected into the dorsal root in dogs failed to abolish the effects of antidromic vasodilatation. Much earlier, Bayliss[7] had noted that when nicotine was painted on the dorsal root ganglion, one could still obtain the characteristic vasodilatation. Such facts led these two observers to conclude that no synapses which were concerned with the vasodilatation phenomenon existed in the dorsal root ganglia.

Obviously direct injection of nicotine into the dorsal root ganglion would produce the best conditions for inhibiting synaptic transmission. Wybauw's method was therefore used on frogs because it was felt that direct observance of arteriolar dilatation, as in the web of the frog's foot, would be a more sensitive way of determining such changes than was Wybauw's use of temperature changes in the paw of the dog's foot.

Method. The frog *Rana pipiens* was used. Under urethane, or after decerebration, the lumbar region of the cord was exposed. In this region the eighth dorsal root and its ganglion can be freely disclosed. A long section of this root is easily obtained if it is severed at its exit from the spinal cord and traced down intraspinally to the ganglion just outside the spinal column. The web of the corresponding foot was so arranged on a piece of cork that it could be observed directly with a microscope under low power. The blood vessels were easily visible and their widths measured by an ocular micrometer. The distal stump of the dorsal root, cut central to the ganglion, was stimulated at its proximal end or in the small portion peripheral to the ganglion with a thyratron stimulator. Leakage of current from the dorsal root into the

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1 Hasama, B. I., *Arch. Int. d. Pharm.*, 1931, **41**, 145.

2 Kuré, K., Saito, S., and Okinaka, S., *Arch. f. d. ges. Physiol.*, 1936, **238**, 290.

3 Ranson, S. W., *Am. J. Physiol.*, 1922, **62**, 383.

4 Ranson, S. W., and Wightman, W. D., *Am. J. Physiol.*, 1922, **62**, 392.

5 Sheehan, D., *Yale J. Biol. and Med.*, 1934, **7**, 425.

6 Wybauw, Lucien, C. R. d. Soc. d. Biol., 1936, **121**, 1377.

7 Bayliss, W. M., *J. Physiol.*, 1900, **26**, 173.

TABLE I.

Tabulation of data indicating changes in the diameters of various blood vessels in the web of the foot of the frog following electrical stimulation of the peripheral end of the cut dorsal root by a thyratron stimulator and the Harvard inductorium before and after injection of the dorsal root ganglion with .25 cc 1% nicotine. The eighth dorsal root in a different specimen of *Rana pipiens* was used in each case. Weak current strengths were used for each stimulation. In the case of the thyratron stimulator, these stimuli were applied at the rate of 2 per second whereas in the case of the Harvard inductorium tetanic stimuli were used.

Vessel	Diameter (μ) before stimulation		Increase in diameter (μ) during stimulation		Stimulator used: Thyratron (T); inductorium (I)
	Without nicotine	With nicotine	Without nicotine	With nicotine	
Large artery	195	—	0	—	I
Small artery	75	—	45	—	I
,, ,,	75	75	15	0	I
,, ,,	60	45	18	0	I
,, ,,	60	45	30	0	I
,, ,,	60	60	11	15	I
,, ,,	60	60	18	0	I
,, ,,	75	75	8	0	I
,, ,,	45	15	15	0	I
,, ,,	75	60	45	0	I
,, ,,	90	90	23	15	T
,, ,,	120	113	45	0	T
,, ,,	105	105	45	0	T
Arteriole	60	60	30	0	I
,,	30	30	15	0	I
,,	75	60	18	15	I
,,	60	45	15	0	I
,,	60	60	22	0	T
,,	30	15	23	0	T
,,	60	—	15	—	T
,,	45	—	15	—	T
,,	30	—	15	—	T
,,	30	15	30	15	T
,,	45	45	30	15	T
,,	45	45	15	0	T
,,	90	90	60	0	T
,,	45	45	15	0	T
,,	45	45	45	0	T
,,	45	45	30	0	T
,,	45	45	15	0	T
,,	45	60	30	15	T
,,	30	30	15	0	T
,,	60	60	30	0	T
,,	45	45	15	0	T
,,	30	60	15	15	T
Vein	60	60	0	0	I

dorsal root ganglion was minimized by employing weak currents produced at a slow rate (the type best for production of parasympathetic-like responses) and by using a sufficiently long piece of dorsal root central to the ganglion. The frog rested on a metal plate which formed the indifferent electrode, whereas the stigmatic electrode consisted of a platinum wire sealed in a small glass tube. This tube was fixed in position near the open cord and the peripheral end of the cut dorsal root was made to adhere to the bit of platinum exposed at the end of this tube. The vasodilatation produced by stimulation of the root central and peripheral to the ganglion was then noted and recorded. This stimulation was performed again in the same manner after the direct injection of .25 cc of 1% pure nicotine into the ganglion and the resultant effects again noted. The changes in width of the observed blood vessel were taken as the criteria for the extent of peripheral vasodilatation.

Results. As shown in Table I, the injection

of nicotine into the dorsal root ganglia of frogs caused complete suppression of the previously elicited vasodilatation. Whereas the average increase in the diameter of small arteries as a result of stimulation of the peripheral stump of the sectioned dorsal root had amounted to 21 μ, it was reduced to O following nicotine injection in 11 out of 13 cases. Similarly, in 17 out of 22 cases of arteriolar dilatation where the average increase in width amounted to 23 μ, there was no change in diameter after nicotine injection into the dorsal root ganglion. Control experiments indicated that the blocking effect of nicotine injection was not due to the pressure of the fluid since injections of .25 cc of 1% atropine sulfate into the ganglion failed to prevent the vasodilator response.

It was further found that during an interval of 2 minutes continuous stimulation of the un-nicotinized dorsal root, the flow of blood in some (5) cases was found to be reduced in the capillaries to 70% of its original rate (number of RBCs per 10 seconds).

To complete the experiment an attempt was made to show that vasodilatation elicited from this portion of the root, peripheral to the ganglion, could still be obtained. In some cases stimulation of the dorsal root peripheral to the nicotinized dorsal root ganglion caused vasodilatation. However, this was usually associated with simultaneous muscular twitching which probably resulted from current leakage to the corresponding ventral root from the very short piece of dorsal root available for stimulation.

Discussion. The results presented in Table I indicate that vasodilatation caused by stimulation of the peripheral end of a dorsal root sectioned between the ganglion and the cord was prevented by the injection of nicotine into the dorsal root ganglion. These results contradict those obtained on dogs by Wybauw. The method of direct injection into the ganglion as described here and as used by Wybauw would seem to be preferable to painting the ganglion (Hasama, Bayliss) or intravenous injection (Kuré) since the nicotine effect would be more localized. The possibility that nicotine may block fine nerve fibers is not excluded since this factor was not controlled. It is a well known fact, however, that nicotine seems to act specifically on synaptic connections so that impulse transmission across the synapse is prevented. The experiments described here provide further definite proof that a synapse is interposed between the vasodilator fibers central to the ganglion and those peripheral to the ganglion. It has been shown previously by Bach[8] that anatomically efferent fibers emerge from the cord and pass peripherally in the dorsal roots. The experiments described here show that physiologically efferent fibers synapse in the dorsal root ganglion. There is no evidence to indicate that the anatomically and physiologically efferent fibers are the same. However, the possibility exists that these fibers are identical and synapse in the dorsal root ganglion.

Conclusions. Evidence is presented to show that synapses exist between physiologically efferent fibers which course the dorsal root in the frog and that these synapses are present in the dorsal root ganglia.

It is further concluded that these synapses are concerned in the transmission of the impulses which result in peripheral vasodilatation since injections of nicotine into the dorsal root ganglia prevent the appearance of vasodilatation which occurs following stimulation of the peripheral stump of the dorsal root sectioned between the ganglion and the cord.

[8] Bach, L. M. N., to be published.

15115 P

Effect of Embryo Extract on Growth of Fibroblasts Previously Irradiated with X-rays.

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Embryo extract is a powerful growth-stimulant for cell colonies *in vitro*. It is generally accepted that its influence is manifested in, and limited to, the activation of cell-division. Our observations on the effect of X-rays on cell cultures make it necessary to revise this view. The investigations reported here demonstrate that cell cultures deprived of mitotic capacity by previous irradiation with X-rays will also grow at a considerably increased rate after the addition of embryo extract.

The experiments were performed on standardized cultures of chicken fibroblasts derived from the hearts of 7-days-old embryos. The cell colonies were cultivated in hanging drops according to the method of Carrel. After the irradiation, they were planted into Carrel flasks. Growth curves of the cultures were constructed from planimetric measurements of outline drawings of the surface areas made every 24 hours. Irradiation was carried out using a demountable X-ray tube which worked at a tension of 35 KV on currents of 20 MA. The tube had a copper anticathode and a window of aluminium foil, 30 μ thick. Absorption analysis showed that the rays penetrating through the window foil and the mica-

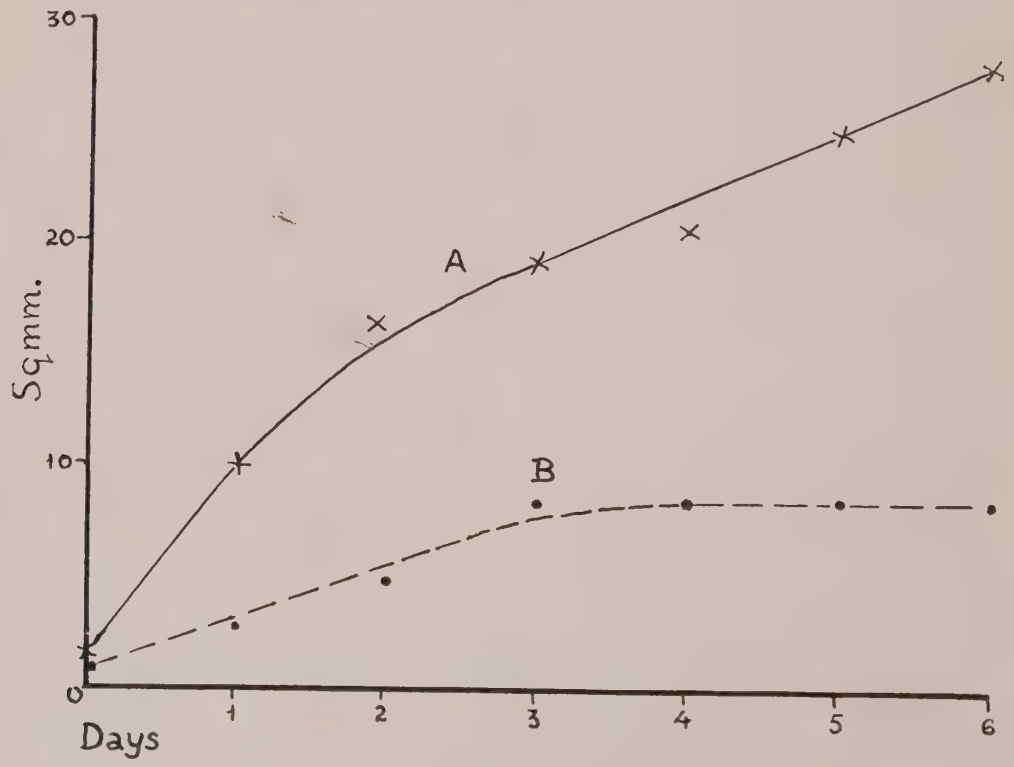

Fig. 1.
Experiment 13108/9b. Growth curves of sister halves of a fibroblast culture irradiated with 25000 r units: A, in medium containing embryo extract; B, in protective medium.

coverglass of the cultures were mainly copper K-rays.

The following procedure was adopted: Preliminary studies having shown that the X-ray dose necessary to inhibit completely cell multiplication is 10,000 r units, cultures of fibroblasts were irradiated with 25,000 r units, *i.e.*, a dose 2½ times greater than that required to arrest all mitoses. The cultures irradiated were divided into two equal parts: one fragment was planted into a medium which consisted of chicken plasma diluted with Tyrode's solution in the proportion 1:2; the other half was cultured in a medium of the same composition to which 30% embryo extract was added as a fluid phase.

In both fragments, the cells deprived by irradiation of their mitotic capacity migrated progressively and surrounded the explant. The growth rate of the culture that developed in a non-growth-promoting medium remained low. But the culture grown in a medium containing embryo extract showed considerable increase in area. After 6 days' cultivation the surface of cultures grown in a medium to which embryo extract was added exceeded by 200% (average of 17 experiments) the growth area of cultures developed in plasma alone. Fig. 1 represents this relation graphically.

The increase in area of a cell colony *in vitro* is the resultant of a number of factors of which the most important are cell multiplication and call migration. The culture in which no cell multiplication occurred enlarged by the migration of cells from explant into the culture medium. The fact that embryo extract promoted the rate of growth in cultures deprived of mitotic capacity proves that embryo extract not only possesses the property of stimulating cell divisions but also increases the migratory activity of the cells. These findings cast doubt on the validity of the idea that embryo extract activates the mitotic capacity of the cells selectively. Rather, they favor the view that embryo extract increases the general activity of the cells (probably by altering the cellular metabolism) and that this activation is reflected in both greater cell mobility and increased mitotic rate of the cells. The recent findings of Willmer and Jacoby[1] on the influence of various concentrations of embryo extract on cells *in vitro* would appear to lend support to this view. By means of cinematographic records, these workers were able to show that increased concentrations of extract caused an increase in the rate of migration as well as in the rate of mitosis of individual fibroblasts.

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15116

Effect of Temperature on the Action of Penicillin* *In vitro*.

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Hobby *et al.*[1] reported that at low temperature penicillin showed very little bactericidal activity, but that this property was enhanced with a rise in the temperature, becoming very marked in that range favoring maximum growth of the organism. Their work showed the interrelation of the bactericidal action of penicillin and rate of bacterial growth. To support a hypothesis that penicillin acts upon organisms during the time of their division. Bigger[2] carried out almost the same type of experiment as the one of Hobby *et al.*, except that his technic of "one loop sterility" allowed the finding to be stated on a semi-quantitative basis. At almost the same time,

* Throughout this paper "penicillin" refers to material containing about 300 units per mg (Merck).

[1] Hobby, Gladys L., Meyer, Karl, and Chaffee, Eleanor, PROC. SOC. EXP. BIOL. AND MED., 1942, **50**, 281.

[2] Bigger, Joseph W., *Lancet*, 1944, **247**, 497.

Lee *et al.*[3] concurred with the above findings and pointed out that under a diversity of conditions, including variations in the temperature, a quantitative relationship was found to hold between the rate of growth of the organisms and the bactericidal activity of penicillin. For instance, if *S. aureus* FDA was kept at 14°C for 7 days, until growth had taken place in the control tubes, definite bactericidal action of penicillin (1 unit per ml) was noted. Eagle *et al.*[4] have found that the spirocheticidal activity of penicillin is greatly enhanced by an increase in temperature, no activity having been found at 8°C, whereas it became increasingly marked up to 39.4°C, the highest temperature studied. Garrod,[5] repeating the work of Hobby *et al.*, Bigger, and of Lee *et al.*, and using data difficult to interpret because of the lack of controls, attempts, on the basis of two observations, to discredit the concept of the relationship of the bactericidal activity of penicillin to the rate of growth of the organism. First, he notes the bactericidal activity of penicillin at 10° and even 4°C in the presence of 10 units per ml of penicillin. This finding, except for the slightly higher concentration of penicillin used (which might possibly alter the results), is at variance with all the work cited above. Secondly, he points out that even though growth ceases at 42°C, the bactericidal action of penicillin is greater at this temperature than at 37°C. We, too, have found that activity is very marked at this temperature, but we have also observed that with *S. aureus* FDA, the rate of growth of the organism may be very pronounced at 42°C. It has thus been clearly shown that with increasing temperature the bactericidal action of penicillin increases. It is now accepted also that by increasing the rate of growth of a susceptible organism, the other conditions remaining effectively constant, there results a faster killing of organisms by a given concentration of penicillin. Since growth rate is so dependent on the temperature of the medium, it has not been an easy experimental task to study the effect of temperature as a single variable on the action of penicillin. We wish to report this effect, among other findings, in this paper.

TABLE I.
Rate of Killing of *Str. agalactiæ* by Penicillin (5 units per ml) at 50°C.

Incubation of culture at 50°C (in min.)	Viable organism per ml in thousands	
	Control	Penicillin present
0	210	210
5	134	106
10	115	67
15	71	46
20	58	25
30	27	14
40	15	6

Direct action of penicillin on organisms at elevated temperatures. Several experiments have been carried out to measure the effect of penicillin on the rate of killing of organisms at temperatures above that at which growth takes place, and in this way to obtain a measure of the direct killing action of penicillin under such conditions. In Table I are shown the results of such an experiment carried out on a strain of *Streptococcus agalactiæ*. In this experiment, a 0.1 ml of a 1-20 dilution of an overnight culture was inoculated into 10 ml of brain-heart broth, giving an initial population of 210,000 organisms per ml. To this, sufficient penicillin Merck was added to give a final concentration of 5 units per ml. A control tube was prepared similarly, but without penicillin. The tubes were allowed to stand at room temperature for about 1 hour before the heating was begun in order to give time for any interaction which might possibly occur between the organisms and the penicillin.

A similar but more complete study of the action of penicillin at elevated temperatures has been carried out on *Staphylococcus aureus* FDA. The conditions were the same in this as in the preceding experiment, and the results are given in Table II.

It is seen from the table that at elevated temperatures 5 units of penicillin per ml kills

[3] Lee, S. W., Foley, E. J., and Epstein, Jeanne A., *J. Bact.*, 1944, **48**, 393.

[4] Eagle, Harry, and Musselman, Arlyne D., *J. Exp. Med.*, 1944, **80**, 493.

[5] Garrod, Lawrence P., *Brit. Med. J.*, 1945, Jan. 27, 107.

TABLE II.
Rate of Killing of *S. aureus* FDA by Penicillin (5 units per ml) at Elevated Temperatures.

Incubation of cultures at given temp. (in min.)	Viable organisms per ml in thousands Temperature of incubation					
	50°C		55°C		60°C	
	Control	Penicillin present	Control	Penicillin present	Control	Penicillin present
0	600	570	600	570	600	570
5	500	450	650	540	46	40
10	450	500	370	320	6.1	2.0
15	450	450	170	150	1.6	0.61
20	420	290	70	38	0.25	0.040
30	450	240	64	24	0	0

TABLE III.
Effect of Rate of Growth of *S. aureus* FDA and of Temperature on the Activity of Penicillin (1.5 units per ml) in Brain-Heart Medium. Effect of Age of Culture.

Age of organisms in inoculum	Incubation of culture at given temp. (in hrs)	Viable organisms in thousands Temperature of cultures					
		37°C		40°C		42°C	
		Control	Penicillin present	Control	Penicillin present	Control	Penicillin present
Overnight	0	1000	1000	1000	1000	1000	1000
	3	5120	540	2400	280	1280	640
3 hrs	0	900	900	900	900	900	900
	3	7840	160	4000	160	3200	145

S. aureus FDA at a much faster rate than does the heat alone. As might be expected, the killing action of the penicillin increases markedly with each rise in temperature.

It was thought of interest to study the effect of slight variation in the incubation temperature near that for optimum growth, as well as the possible effect of using inocula of an organism of different ages. In a typical experiment, 0.1 ml of a 1:50 dilution of an overnight growth of *S. aureus* FDA was inoculated into a tube of brain-heart medium containing 1.5 units of penicillin per ml. For the young culture, 0.1 ml of a 3-hour growth of the same organism was inoculated into another tube of the above medium containing penicillin (1.5 units per ml). These results are given in Table III.

It has been amply and quantitatively shown that, other conditions remaining as constant as possible, the rate at which penicillin kills susceptible organisms increases with the rate of growth of the organism. This quantitative data has previously not been extended beyond the temperature range favoring maximum growth. The representative data given in Table III are then of interest, for they indicate that at temperatures higher than 37°C the viable count in the presence of penicillin decreases at a faster rate, even though rate of growth in the control tubes is diminished. Thus, penicillin action either increases with temperature as such, or its action is a function of the rate of the metabolic processes of the organism.

An experiment similar to the previous one was carried out using rabbit serum as a medium. This was done to see whether the temperature-activity relationship would hold under these conditions. On the basis of the results of preliminary experiments, such as those given in Table IV, it appears that an increase in temperature does result in an increase in the killing action of penicillin, but the findings are not as definite as in the simpler media.

Summary. At elevated temperatures (50-60°C) penicillin (5 units per ml) hastens

TABLE IV.
Effect of Rate of Growth of *S. aureus* FDA and of Temperature on the Activity of Penicillin (1.5 units per ml) in Rabbit Serum.

Incubation of cultures at given temp. (in hrs)	Viable organisms per ml in thousands Temperature of Incubation					
	37°C		39°C		42°C	
	Control	Penicillin present	Control	Penicillin present	Control	Penicillin present
0	120	120	120	120	120	120
2	850	600	2700	550	800	120
4	11000	30	17000	10	46000	5.0

the death of *S. aureus* FDA and a strain of *S. agalactiæ*. At temperatures higher than that leading to maximum growth of the organism, penicillin kills *S. aureus* FDA faster than it does at the temperature of maximum growth. At the temperatures slightly higher than optimum, there still exists an interrelation between growth and the killing of bacteria by penicillin. It is believed that only at excessively high concentration, or very high temperature, does penicillin kill organisms while they are not undergoing growth.

15117

Use of Beta Beta' Dithiocyano Diethyl Ether (RID-O) to Control Mite Infestations in Mice.*

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When it was noticed that part of a colony of 3,000 mice used for cancer research were losing hair, they were examined for mites. The skin on the abdomen was scraped with a small sharp scalpel on which a drop of glycerine had been placed. The scrapings were then put in a drop of glycerine on a glass slide and examined with a microscope. Glycerine was used because mites remain active in this medium for many hours. The species of Acarina (mites) found was identified as *Myobia musculii*.

Since rotenone and derris root powder were not available, an attempt was made to kill the mites with DDT.† The results were disastrous because the DDT powder was much more toxic for mice than it was for mites. Some papers on the toxicity of DDT for mice, rats, cats, etc., appeared about this time and confirmed the observation that it is toxic for mice.[1,2,3] Animals that lick their fur and thus ingest agents applied externally, cannot be treated with DDT. In addition to the toxicity, DDT was of no value in this case because it did not appear to kill the mites. This lack of lethal effect of DDT on mites may be related to the fact that mites are not insects, but arachnids.

The next insecticide tried was a liquid

* This work was assisted by grants from The Anna Fuller Fund and The International Cancer Research Foundation.

† A small sample of DDT was very kindly supplied by the Research Department of the School of Pharmacy of the University of Maryland.

1 Lillie, R. D., and Smith, M. I., *Public Health Rep.*, Washington, D.C., 1944, **59**, 979.

2 Nelson, A. A., Draize, J. H., Woodard, G., Fitzhugh, O. G., Smith, R. B., Jr., and Calvery, H. O., *Ibid.*, 1944, **59**, 1009.

3 Woodard, G., Nelson, A. A., and Calvery, H. O., *J. Pharm. and Exp. Therap.*, 1944, **82**, 152.

(TOXITE) designed for the treatment of red mites and insects infesting poultry and other animals. The label states that this is a mixture of xylenols, para cresol, meta cresol, mineral oil, and hydroxytoluenes. This was sprayed on the mice, and while it seemed to kill the mites with which it came in contact, it also killed some of the mice. About this time, cubé root, a substitute for derris root, was obtained. This supposedly contained 5.2% rotenone and it was assumed that it would be no more toxic than derris root. This proved to be an erroneous assumption, because when 300 mice were dusted with this material, 50 of them died during the next 3 days.

Finally, after all these unfortunate experiences with rodenticidal insecticides, a material was found that is extremely lethal for mites and relatively non-toxic for mice. The purpose of this paper is to describe its use and discuss its toxicity in the hope that this information will be of value to others who use mice. This may also prove to be applicable to other animals, but it has not been extensively tested. It has been used on rabbits, guinea pigs, and dogs without noticeable toxicity. A dog was effectively cleared of fleas.

The material found to be effective in controlling mites is a recently developed commercial roach, ant, and bedbug powder called RID-O‡ which contains as the active ingredient 7% beta beta' dithiocyano diethyl ether. Before application to the mice, this product was diluted with 1 to 1½ times its weight of powdered talc. This decreased the tendency to form clumps, and, therefore, the powder was more easily blown into the fur. The mice, the wood shavings, and the container in which the mice were kept were all dusted. The blower or dusting device was so designed that it was not necessary to remove the mice from the glass bowls or cages for the treatment. Large numbers of mice could, therefore, be treated in a relatively short time.

The dusting device essential for the successful application of this powder mixture was constructed from a bottle with a two-hole rubber stopper and several pieces of rubber and glass tubing.§ This was attached to an air jet which was adjusted so that the stream of air which carried the powder was just sufficient to ruffle the fur of the mice. If excess amounts of powder accumulated on the mice, this was partially blown off by a stream of compressed air which circumvented the powder bottle. It was desirable and almost necessary to carry this operation out in a chemical hood for the powder, when inhaled, was found to be irritating to the mucous membranes.

This system has many advantages over usual methods of treating mice with powders. When mice were individually dipped in a powder bowl, the eggs, which were not killed, came off into the powder. Mite-free mice subsequently treated, picked up some of these eggs and thus became infested with mites. With the method described in this paper, such spreading of mites or their eggs is not possible.

When mite-infested mice were treated with RID-O as described above, the mites died in less than 2 minutes, or before they could be scraped off and examined under a microscope. A comparative study of the lethal effect of DDT and RID-O on mites was therefore made. Two sets of mites from the same animal were placed on glass slides. One set was treated with RID-O and examined immediately. The mites stopped moving in about one minute after application. The other set of mites was given a heavy treatment of 10% DDT in talc. These DDT-treated mites floundered about in this powder for several hours. DDT may eventually be lethal for mites, but even this is doubtful as living mites can usually be isolated from mice treated with this substance. In contrast to this, living mites have not been isolated from mice treated with RID-O.

The one unsatisfactory feature of the RID-O treatment is that it does not kill the eggs or larvæ. It is, therefore, necessary to repeat the treatment at appropriate intervals if the mites are to be eliminated from the colony. That RID-O is practically non-toxic was

‡ We are indebted to the Leeds Chemical Co., 509 Pratt St., Baltimore 1, for a generous supply of RID-O.

§ Space limitations prevent a more detailed description which will be supplied by the author on request.

TABLE I.
Toxicity of Insecticides Applied to Mice. Twenty Mice in Each Group.

Group	Treatment		No. of mice dying during intervals indicated (weeks) 1	3	5	7*	9	11	% dead	15	19	23
1	RID-O	daily	0	0	0	0	0	0	0	Discontinued.		
2	"	biweekly	0	0	0	1	0	0	5	Mice used for RID-O		
3	"	weekly	1	0	0	0	0	1	10	feeding test.		
4	"	monthly	0	0	0	1	0	0	5	See Table II.		
5	TOXITE	daily	1	2	1	1	0	0	25	0	0	0
6	"	biweekly	0	0	0	1	4	0	25	1	1	0
7	"	weekly	0	0	0	3	0	0	15	0	0	0
8	"	monthly	0	1	1	0	0	0	10	0	0	0
9	DDT	daily	0	1	1	4	2	0	40	0	0	0
10	"	biweekly	0	1	0	0	2	1	20	1	0	0
11	"	weekly	0	1	1	0	0	0	10	0	2	1
12	"	monthly	0	1	0	0	0	0	5	0	3	2

* Treatment stopped at end of 8 weeks.

TABLE II.
Toxicity of RID-O When Mixed with the Diet.

Group	No. of mice	Fed RID-O	Amt* per 3 g diet	Deaths during intervals indicated—in weeks 1	2	3	4	6
1 A	10	3 wks	1 mg	1	1	1	0	0
B	10	1 day	1	0	0	0	0	0
2 A	8	3 wks	10	1	2	0	0	0
B	10	1 day	10	0	0	0	0	1
3 A	8	3 wks	5	0	0	0	0	0
B	10	1 day	5	3	0	0	0	0
4 A	10	3 wks	25	1	3	0	0	0
B	9	1 day	25	0	0	0	0	0

* The amounts in this column refer to amounts of active ingredients in the RID-O.

established in several experiments. In one of these, the relative toxicity of DDT, TOXITE, and RID-O was tested by treating one group of 60 mice (20 in each set) with these agents daily except Sundays, for a period of 8 weeks. In 3 other groups, the mice were treated biweekly, weekly, and monthly, respectively.

The results are shown in Table I. In the mice treated daily with RID-O for 8 weeks, none of the 20 mice died. Five (20%) of the mice treated daily with TOXITE died, while 8 (40%) of the mice treated daily with DDT died. In the groups of mice treated less frequently, the deaths due to TOXITE and DDT were not so numerous.

At the end of 11 weeks, the animals that had been dusted with RID-O were fed diets containing various amounts of RID-O. In general, each of the former groups was divided in 2: one was fed RID-O for 3 weeks; the other was fed the RID-O diet for only one day. In Groups 2 and 4, it may be seen in Table II, there was some mortality. The RID-O diets in these cases contained 10 mg and 25 mg of beta beta' dithiocyano diethyl ether per 3 g of diet. These concentrations were so high that the animals refused to eat and some of these deaths were attributed to starvation rather than to the toxicity of the substance. The results of this experiment were very inconsistent as seen by Table II. There is some indication that a tolerance to the drug can be built up and that the natural resistance of the animals varies considerably.

Summary. Beta beta' dithiocyano diethyl ether (RID-O) has been found to be very effective in controlling mites and exhibits a relatively low toxicity in mice. This chemical may be found useful as an insecticide or an acaricide on other laboratory and domestic animals.

15118 P

Absence of Alteration in the E.E.G. with Stilbesterol and Progesterone.

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Dusser de Barenne and Gibbs[1] in 1942 presented evidence to indicate that the E.E.G. was modified during the menstrual cycle. Gibbs and Reid[2] (1942) demonstrated that in the last weeks of pregnancy the electrical activity of the cortex was definitely slowed compared to postpartum E.E.G. records. Since it is well known that in both menstruation and pregnancy marked changes occur in the blood levels of follicular and corpus luteum hormones, it was decided to determine whether E.E.G. changes could be produced by the administration of stilbesterol and progesterone to women in the menopause.

In order that neither the changes during the menstrual cycle nor during pregnancy might interfere with the electrical activity of the cortex three women in the menopause were chosen for study. All three were patients in the Boston Psychopathic Hospital suffering from agitated depressions of moderate severity. None of the patients complained of menopausal symptoms.

Daily E.E.G.'s were obtained with the aid of a Grass six-channel instrument throughout the period the hormones were given. The electrodes were applied to the frontal, motor, and occipital leads bilaterally and referred to indifferent interconnected ear leads. Before the administration of a hormone preparation E.E.G.'s were obtained from each patient during a control period of 7-14 days.

Case 1 was a 53-year-old patient who passed through menopause about 12 years ago. She received increasing doses of stilbesterol by mouth varying from .25 to 10 mg daily for 53 days. During the last 10 days of this 53-day period intramuscular injections of progesterone (10 mg in 1 cc ampules) were administered daily. The administration of the hormones was discontinued because the patient began to menstruate.

Case 2 was a 49-year-old patient who had not menstruated for 7 months. She received daily injections of progesterone without stilbesterol for 39 days and then both hormones were given together for 20 days. The progesterone was given in the same dosage as in Case 1 and the stilbesterol was gradually increased to 10 mg daily. This patient menstruated for 9 days during the administration of progesterone alone.

Case 3 was a 53-year-old patient who had passed through the menopause about two years before. She received stilbesterol and progesterone for a period of 20 days in the same dosage as the above two cases. The patient stated that she menstruated though this was not definitely confirmed.

All patients had normal E.E.G.'s before medication. None of the patients showed significant changes in the E.E.G. at any time during the period the hormones were administered.

Apparently large doses of stilbesterol and progesterone hormones given alone and together to the non-pregnant, non-menstruating, menopausal woman are without effect on the cortical electroencephalogram. These hormones evidently exercised a profound influence on the endocrine balance of these women since two of the three menstruated during the course of medication. This does not exclude the possibility that the effects obtained by Gibbs, de Barenne, and Reid were due to hormonal influences but strongly suggests that factors other than endocrine should be considered.

1 de Barenne, D., and Gibbs, F. A., *Am. J. Obs. and Gyn.*, 1942, **44**, 4-687.

2 Gibbs, F. A., and Reid, D. E., *Am. J. Obs. and Gyn.*, 1942, **44**, 4-672.

* Lutocylin obtained through the courtesy of Ciba Pharmaceutical Products, Inc., from Dr. Ernst Oppenheimer.

15119

The Cephalin-Cholesterol Flocculation Test in Malaria.

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This study was done as a result of a report[1] stating that all patients with malaria had an abnormal liver function as tested by the cephalin-cholesterol flocculation test. Subsequently, in a report on phlebotomus fever,[2] Sabin *et al.* reported that in sand fly fever this test is negative and that the test can be used as an aid in differential diagnosis between that disease and malaria.

All our tests were done with a commercial preparation of cephalin-cholesterol and, at the start, readings were taken at 24 and 48 hours. In the middle of this series it was noted that some results apparently did not follow consistently if the 48-hour reading were used. The 24-hour reading appeared to give more consistent results. A series of tests were then performed on 20 psychotic patients. These produced negative results at 24 hours and positive reactions if the 48-hour readings were used. This has been previously reported.[3] Other factors tending to increase false positive reactions are light and temperature. More consistent results were obtained when tests were incubated in the dark at room temperature.

Our first series consisted of 114 patients and our second series of 102 patients. The tests were done within 24 to 48 hours after admission for a malarial attack and was repeated once a week, when possible, until the patient was discharged from the hospital.

Table I shows the result of the 2 series. It will be noted that if only one reading were taken, on admission, even with the reading of 48 hours, only 52% of the results were positive. If the 24-hour reading were used, more of the patients showed negative results. It is to be noted that Sabin *et al.* used the 24-hour reading in their report. When follow-up tests were done, as recorded in section marked "2 or more readings," a greater percentage showed positive results. In the 48-hour readings, 58.6% had a positive reading on admission and an additional 34.3% became positive during their stay. Only 7.1% remained negative. If 24-hour readings were used, the percentage that remained negative was 24.3%. When 24-hour readings only were done, the percentage that remained negative was essentially the same as in the 24-hour group of series I.

TABLE I.
Cephalin-cholesterol Flocculation Results in Malaria.

	Series I				Series II	
	Read at 48 hr		Read at 24 hr		24-hr technic	
	No.	%	No.	%	No.	%
One reading:						
Pos. on Adm.	23	52	17	38.6	4	36.4
Neg. on Adm.	21	48	27	61.4	7	63.6
Two or more readings:						
Pos. on Adm.	41	58.6	23	32.85	32	35.2
Pos. sometime	24	34.3	30	42.85	35	38.4
Negative	5	7	17	24.3	24	26.4

1 Mirsky, I. A., Von Brecht, R., and Williams, L. D., *Science*, 1944, **99**, 20.

2 Sabin, A. B., Philip, C. B., and Paul, J. R., *J. A. M. A.*, 1944, **125**, 693.

3 Pohle, J. F., and Stewart, J. K., *J. Clin. Invest.*, 1941, **20**, 241.

We could not find any correlation between the results of the tests and the number of attacks of malaria that the patient has had. Two soldiers came back with a subsequent attack while the study was going on. One had had a positive series of tests on the first admission and a negative series on the second. Another had negative tests on both occasions. There was also no correlation between the duration of the malaria, dated from the first attack, or between the origin of the malaria and the result of the cephalin-cholesterol flocculation test.

As stated by Mirsky[1], there was no correlation between the result of the Kahn test and the cephalin-cholesterol flocculation test, regardless of time of reading of the test.

These results do not verify the statement that all patients with malaria show impairment of liver function as measured by the cephalin-cholesterol flocculation test. The 24-hour technic shows a lower percentage of positive results than the 48-hour technic. The percentage of positive results becomes higher if repeat tests are done, but never higher than 75%. The cephalin-cholesterol flocculation test is not an absolute differential diagnostic point between malaria and sand fly fever.

15120

Further Observations on Red Cell Agglutinating Agent Present in Lungs of Virus-Infected Mice.

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In a previous communication[1] we have reported the presence of a heat-stable hemagglutinating agent in the lungs of mice infected with a pneumonitis virus. This agent agglutinates murine erythrocytes and the agglutinin is inhibited by specific antiserum. The agglutinating property becomes manifest only after treatment of infected lung extract by heat.

The work reported in this paper, and the previous one, has been carried out with the "ES" strain of pneumonitis virus, a strain nearly identical with the pneumonia virus of mice, described by Horsfall and Hahn.[2,3] The hemagglutinating agent is regularly present in the lungs of infected mice. It has been demonstrated in mice killed 48 hours after intranasal inoculation, is present in its highest titer 5-6 days after infection and is usually absent in mice surviving 9-10 days or longer.

Preparation of the agglutinating lung extract has been simplified. Each pair of infected lungs is ground with sand and 2 cc of normal salt solution. The suspension is heated for 10 minutes in a water bath at 75-80°C, centrifuged, and the supernatant fluid removed for study. Various tests have been performed in order to determine the nature of the agglutinating agent and the conditions under which the agglutinating property is masked or unmasked.

The results in Table I indicate that the max-

TABLE I.
Range of Temperature Causing Maximum Activity of Agglutinating Property of Lung Extract.

Degree of heat °C	Time, min.	Agglutination (titer)
Untreated	—	0
56	30	0
60	15	±
65	5	±
70	5	1-64
75	5	1-64
80	5	1-32
85	5	1-16
90	5	±
95	5	trace
100	5	0

1 Mills, K. C., and Dochez, A. R., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 140.

2 Horsfall, F. L., and Hahn, R. G., PROC. SOC. EXP. BIOL. AND MED., 1939, **40**, 684.

3 Horsfall, F. L., and Hahn, R. G., *J. Exp. Med.*, 1940, **71**, 391.

imum agglutinating power of the extract develops after heating for 5 minutes at temperature ranges from 70°-80°C and that the agent is remarkably resistant to heat.

The red cells of a number of animal species have been examined for their agglutinability. Those yielding positive results are the red cells of the mouse and the hamster. Those yielding negative results are human group 0, fowl, guinea pig, rat, cotton rat, sheep, cat, dog, and ferret. Rabbit cells show slight but irregular agglutination.

The agglutinating agent, in the state in which it exists in fresh lung suspensions, retains its activity for at least 7 days at room and at ice-box temperatures. Heated extracts prepared from such preserved suspensions exhibit undiminished agglutinating capacity.

The inability of fresh unheated lung extracts to cause agglutination of mouse red cells seems to be due to masking of its activity by substances derived from blood or lung tissue in the process of maceration by grinding. In order to test this hypothesis substances which might be present in fresh mouse lung were added to a heated agglutinating extract and their capacity to affect the agglutination phenomenon was observed. The addition of normal mouse serum to heated agglutinating extract resulted in no diminution of agglutinating capacity. On the other hand, when washed murine red cells were added to such an extract, allowed to stand for 1 hour at room temperature, and the mixture then centrifugalized and the supernatant fluid tested for agglutinating capacity, this power was either completely lost or markedly diminished. High concentrations of red cells 33-40% effected complete loss, 5-10% very marked diminution and 1.25-2.5% moderate to slight diminution of the agglutinating power of the extract. Removal of the cells by centrifugation and retreating of the supernatant fluid at 80°C did not result in restoration of the agglutinating capacity. Treatment of fresh lung suspensions in the same manner resulted, upon removal of the red cells, in retention of undiminished infectivity and the development of a high agglutinating titer by the extract after heating. In short, the addition of a large volume of suitable red cells to heated agglutinating extract completely absorbs out the agglutinating agent, whereas a similar procedure applied to untreated lung suspensions leaves, upon subsequent heating, the agglutinating capacity unchanged.

The agglutinating agent present in heated lung extract can also be completely absorbed and removed by means of laked murine red cells, mouse stromata, and by ground up fresh normal mouse lung.

Other methods of preparing an active agglutinating extract such as: thorough perfusion of the lung before removal, exposure of the suspended lung tissue to 15% sodium chloride, 5% formalin, 1% calcium chloride, half saturation with ammonium sulfate, and 50% alcohol did not result in the development of agglutinating power by the lung tissue suspension.

Horsfall and Hahn have reported that mice can be actively immunized against the pneumonia virus of mice by intraperitoneal injections of living virus. It seemed of interest therefore to determine if the heated virus-containing extract discussed above has the capacity to induce active immunity in mice. Mice were given at 4-day intervals three injections of 0.5 cc of heated extract. Seven days after the last injection the mice were challenged by intranasal inoculation of a 50% mortality dose of living virus. All mice previously injected with extract were solidly immune, whereas all controls developed characteristic lung infections. Furthermore, the serum of mice immunized against the extract inhibited to high titer agglutination of red cells by active extract. In our stock, normal mouse serum possesses no such inhibitory power.

Rabbits immunized with living E.S. virus or the standard pneumonia virus of mice rapidly develop antibodies capable of neutralizing the agglutination reaction. Serum of a rabbit immunized with heated lung extract neutralized the agglutinating power of the extract at high titer and the infectivity of living virus at a somewhat lower level.

Horsfall[4] has noted the infectivity of pneumonia virus of mice for hamsters. Inoculation of mouse lung suspensions containing

[4] Horsfall, F. L., Personal communication.

the E.S. virus into the Syrian hamster frequently causes large areas of pulmonary consolidation and occasionally death of the animal. Serial passage, with irregular exceptions, could not be successfully achieved. Agglutination of red cells exuding from consolidated hamster lungs was regularly observed and heated extracts of these lungs agglutinated both mouse and hamster red cells in high titer. Normal serum from these hamsters had a low capacity for inhibiting red cell agglutination, whereas the serum of animals recovering from infection acquires this power readily. In contrast, the serum of recovered mice acquires the capacity for agglutinin-inhibition more slowly—about 9 days after infection. It is possible that the early development of immunity by the hamster interferes with the spread of infection in this animal.

Dr. David E. Green and Miss Violet Nocito have undertaken the purification of the agglutinating agent.

The agent was readily purified by successive precipitations with ammonium sulfate. At 20-35% saturation of ammonium sulfate inactive proteins were removed whereas the agglutinating agent precipitated out in the saturation range 35-50%. Solutions thus purified were colorless and clear, and were approximately 15 times as active as the original heated extract per unit of protein. The immunizing capacity of the agglutinating agent tested at this purity level was found to be unimpaired. The studies on the purification of the agglutinating agent are being continued and will be reported elsewhere.

Summary. The red cell agglutinating agent present in the lungs of mice infected with pneumonitis virus has been subjected to further study. Maximum agglutination develops after heating the fresh lung extract at 75°C for 5 minutes. The agent is absorbed from active extracts by preparations of mouse red cells and of fresh normal mouse lungs. Mice injected with the heated agent develop active immunity against the living virus and their sera have the capacity of inhibiting red cell agglutination. The sera of rabbits immunized against living pneumonitis virus or against active heated mouse lung extract inhibit red cell agglutination. Hamsters appear to be susceptible to infection with the pneumonitis virus but attempts at continued serial passage were unsuccessful. The agglutinating agent has been purified by precipitation with ammonium sulfate. The purified agent retains its agglutinating and immunizing capacity.

15121

Artificial Induction of Subcutaneous Nodules in Rheumatic Fever.*

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From the A.A.F. Rheumatic Fever Control Program.

During the course of studies on various aspects of rheumatic fever a working hypothesis was developed which postulated that this syndrome is one of the manifestations of the uninhibited action of proteinases on mesenchymal tissues. It was proposed that these proteolytic enzymes are either activated *in vivo* by streptococcal fibrinolysins or result from the release of intracellular proteinases consequent to the cellular damage produced by infection, trauma, or "anaphylactoid" reactions. Further, it was assumed that the individual who is susceptible to the development or recurrence of rheumatic fever is one who cannot inhibit adequately the proteinases thus activated. The present report is concerned with the application of this hypothesis to a study of the induction of subcutaneous nodules in susceptible subjects.

Although Drewitt[1] postulated in 1883 that trauma was involved in the production of subcutaneous nodules in rheumatic fever, it

[1] Drewitt, F. D., *Brit. M. J.*, 1883, **1**, 622.

was not until 1937 that evidence for this concept was reported. At that time Massell, Mote and Jones[2] demonstrated that the injection of 2 to 3 cc of whole blood subcutaneously over the anesthetized olecranon process, with subsequent frictional pressure, resulted in the appearance of a nodule in the injected region in 90% of those patients with clinically active rheumatic fever, in 50% of those with only laboratory evidence of active rheumatic fever and in only 14% of those rheumatic fever patients who had no evidence of active disease. Accordingly, these investigators suggested that tissue injury, if sufficient, may be of primary importance in the spontaneous production of a nodule in the patient with acute rheumatic fever, though some specific effect of blood itself could not be ruled out.

It is apparent that the destruction of cells by trauma will result in the release of their intracellular enzymes, which, if not inhibited by proteinase-inhibitors, may in turn produce the secondary changes responsible for the appearance of the nodule. Accordingly, we studied the influence of the subcutaneous injection of trypsin on the induction of a nodule. It must be emphasized that although trypsinases are known to be present in cells, it is quite possible that some other proteinase may prove to be involved.

Four groups of male subjects of from 19 to 40 years of age were investigated. The first group consisted of men who were recovering from a variety of clinical disturbances but who gave no personal or family history suggestive of rheumatic fever and did not present any evidence of a recent streptococcal infection. They are designated as "non-streptococcal controls." The second group was comprised of subjects who had had B hemolytic streptococcal infections (as proven by positive cultures) 2 to 3 months before the present study. None of these patients developed any indications of rheumatic fever, and are designated as "streptococcal controls." The third group was made up of subjects who gave a definite personal or family history of rheumatic fever, but were hospitalized, at the time of the study, for some other reason. This group is designated as "rheumatic fever history." The fourth group consisted of men who had developed signs and symptoms of rheumatic fever but were convalescing at the time when the present study was performed.

The majority of subjects were injected subcutaneously over the ulna with 1 cc of 1% solution of crystalline trypsin magnesium sulfate compound in either saline, M/400 HCl, or water, while a number of patients were injected with 1 cc of a 5% solution of crude trypsin (Wilson). All solutions were filtered (Seitz) before use. No difference was noted between the effects of the crystalline and crude preparations except that a concentration of less than 5% of crude trypsin was ineffective.

Edema usually developed at the site of the injection in from 2 to 3 hours and in some instances (usually the rheumatic fever subjects) persisted for as long as 48 hours. Those subjects who developed nodules usually developed the lesion at the site of injection in from 4 to 7 days. Thus some subjects showed no evidence of a real nodule until the 7th day after the injection, while others developed one by the 4th day. The nodule had a definite contour and was readily distinguished from the indefinite induration which occurred at the site of injection in about one-third of all subjects. In the proper light, the nodule was easily detected.

The size of the induced nodules varied a great deal but this feature could not be related to activity nor to any clinical or laboratory observation. The duration of the nodules also varied markedly: from a few days to many weeks.

The subcutaneous injection of trypsin did not induce any change in either blood morphology or erythrocyte sedimentation rate. A number of rheumatic fever patients volunteered the information that they experienced fleeting pain in various joints during the first 48 hours after the injection, but the significance of this cannot be evaluated at the present time.

The preliminary data are summarized in Table I and suggest that subjects who are convalescing from rheumatic fever or those who have had a personal or family history of this syndrome respond to the subcutaneous injec-

[2] Massell, B. F., Mote, J. R., and Jones, I. D., *J. Clin. Invest.*, 1937, **16**, 126.

TABLE I.
Induction of Subcutaneous Nodules with Trypsin.

Group*	Total No.	Positive	
		No.	%
Non-streptococcal controls	75	10	13.3
Streptococcal controls	40	1	2.5
Rheumatic fever history	22	17	77.3
Rheumatic fever convalescents	145	102	70.3

* See text.

tion of trypsin with the production of a nodule. The fact that 13% of the "non-streptococcal controls" developed nodules while 30% of the "rheumatic fever" subjects did not do so may reflect an inadequate history or a faulty diagnosis.

It is of interest to note that of the 40 patients who had had a previous streptococcal infection, without developing rheumatic fever, only one developed a nodule at the site of the trypsin injection. This observation is subject to a variety of interpretations. Thus it may be inferred that since this group of patients did not develop rheumatic fever, in consequence of their streptococcal disease, they represent subjects who are not "susceptible" to the disease. On the other hand, since a sulfonamide resistant strain of (Type 17) B hemolytic streptococci was isolated from the majority of these patients, it is possible that they were not "sensitized" to some streptococcal factor present in other strains of the organism. However the fact that infection with this strain of streptococci has been followed by rheumatic fever in some cases that have been reported suggests that a lack of streptococcal sensitization is not responsible for the negative results.

The specificity of the response to trypsin must await further study. Thus a small group of patients with rheumatoid arthritis behaved like the rheumatic fever group. This may indicate that both diseases are different manifestations of one disorder, as is suspected by many investigators, or may reflect the nonspecificity of the response to trypsin. However, it is of importance to note that since the appearance of a nodule is not related to the presence of active disease, it is improbable that we are dealing with some general sensitization but that the response may be related to whatever phenomenon is responsible for the susceptibility to rheumatic fever.

A few experiments with trypsin which was previously inactivated with "trypsin-inhibitor" suggest that the response noted above is due specifically to the trypsin. Further, our data indicate that the nodules produced by Massell, Note, and Jones are not due to the blood which they injected but probably to the tissue damage that was thereby induced. However, more extensive studies must be made before any conclusions can be reached as to the validity of the hypothesis outlined at the outset, and as to whether the induction of subcutaneous nodules has any prognostic or diagnostic significance.

Summary. The subcutaneous injection of a solution of trypsin induced "nodules" at the site of injection in 70% of patients convalescing from rheumatic fever while only 13% of apparently normal subjects responded in a similar manner.

15122

Effect of Vitamin K Deficiency on Liver Lipids in the Chick.*

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In a series of recent communications[1-4] Honorato and his associates have indicated that 2-methyl-1,4-naphthoquinone possesses lipotropic properties analogous to choline and suggest that the former substance serves as a "methyl-donor." They report that avitaminosis K in the chick is accompanied by a fatty degeneration of the liver which is prevented by the administration of 2-methyl-1,4-naphthoquinone as well as by choline.[4] These results are at variance with the early observations of Dam and his group,[5,6] as well as other laboratories,[7] in the studies which led to the isolation and identification of vitamin K. These previous studies did not reveal any correlative pathological change in deficient chicks other than alterations resulting exclusively from the characteristic hemorrhagic syndrome. If the absence of vitamin K produces fatty liver degeneration, a route through which the naphthoquinone may function to maintain normal plasma prothrombin levels is suggested. In a previous paper Emmel and Dam[8] reported that no histological indication of fatty degeneration could be seen in the livers of chicks reared on a vitamin K-free diet. The trials herein reported present an elaboration of this study[8] and the amount of total lipid and phospholipids in the liver as determined by chemical methods is given.

Methods. One-day-old White Leghorn chicks were placed on a basal synthetic diet consisting of: alcohol-extracted casein, 150 g; dried brewer's yeast, Fleischmann Type 2019 (ether extracted), 75 g; gelatin, 80 g; McCollum's salt mixture No. 185 with Mn

TABLE I.

	Basal synthetic diet	Basal plus choline	Basal plus vit. K	Control natural diet
Prothrombin levels in 4-week-old chicks.				
Avg clotting time, sec.	106.6	73.0	20.5	20.2
Range	60.0->200	46.2-91.3	16.5-30.0	20.0-20.5
Total lipids in livers of 4-week-old chicks.				
Lipids in % wet wt. of liver, avg	4.93	4.08	5.23	3.64
Range	3.51-7.00	2.79-5.34	3.25-7.75	3.43-3.94
Lipids in % dry wt of liver, avg	17.68	16.57	18.34	14.05
Range	13.48-25.22	12.55-19.70	12.32-25.60	13.75-14.28
Total phospholipid (calculated as P) in livers of 4-week-old chicks.				
Mg P in $CHCl_3$ extr. per g wet liver, avg	.85	.80	.82	.80
Range	.76-93	.66-.95	.65-.93	.76-.86
Mg P in $CHCl_3$ extr. per g dry liver, avg	3.08	3.17	2.89	3.09
Range	2.73-3.67	2.41-3.84	2.06-3.32	2.72-3.44

* Aided by a grant from Wyeth, Inc., Philadelphia. We are indebted to Hoffmann-La Roche, Inc., for supplying the *d,l*-alpha-tocopherol acetate and Synkayvite (tetra sodium salt of 2-methyl-1,4-naphthohydroquinone diphosphoric acid) used in these studies.

1 R. Honorato C., and V. Garcia Merino, *Rev. med. y. alimentacion.*, 1942-3, **5**, 139.

2 R. Honorato C., and A. Cassis, *Rev. med. y. alimentacion.*, 1942-3, **5**, 141.

3 R. Honorato C., N. Ivanovic F., and H. Palma, *Commun. Soc. Biol. Chile*, 1944, **9**-V.

4 S. Topelberg G. and R. Honorato C., *Rev. Soc. Argentina Biol.*, 1943, **19**, 409.

5 Schonheyder, F., Thesis, 1936, University, Copenhagen.

6 Dam, H., Schonheyder, F., and Tage-Hansen, E., *Biochem. J.*, 1936, **30**, 1075.

7 Butt, H. R., and Snell, A. M., *Vitamin K*, 1941, Philadelphia.

8 Emmel, V. M., and Dam, H., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 11.

and Cu added, 50 g; gum arabic, 50 g; *l*-cystine, 1 g; cod liver oil, 50 g; *d,l*-alpha-tocopherol acetate, 50 mg; and cornstarch, 544 g. This diet was supplemented either with 5 g choline chloride (replacing 5 g cornstarch) or 30 mg of a vitamin K compound, the tetra sodium salt of 2-methyl-1,4-naphthohydroquinone diphosphoric acid (Synkayvite, Hoffmann-La Roche). After 2, 3, and 4 weeks blood samples were withdrawn by cardiac puncture into sodium oxalate solution without anesthetic. The prothrombin time of the plasma was determined by adding 0.2 ml of a mixture of $CaCl_2$ and an emulsion prepared from brains of vitamin K-deficient chicks to 0.1 ml oxalated chick plasma at 38°C. Plasma fibrinogen was determined by a modification of the colorimetric method of Folin and Ciocalteu.[9] The chicks were sacrificed after 4 weeks by decapitation and the livers removed. Livers not taken for immediate analysis were frozen. Total lipids were determined by extraction with chloroform after preliminary grinding of the liver with sodium sulfate. The phospholipid content was measured by oxidizing the lipids obtained from a 10 ml chloroform aliquot with sulfuric acid and hydrogen peroxide (Superoxol, Merck) and determining the P- content by a standard colorimetric procedure.[10]

Results and Comment. In all cases determinations were made on groups consisting of an average of 9 (5 to 12) chicks. The plasma prothrombin time of chicks receiving either the synthetic diet supplemented with vitamin K or a commercial growing chick ration was approximately the same while the prothrombin time from chicks on the synthetic diet, with or without added choline, was markedly prolonged (Table I). The plasma prothrombin time from chicks receiving added choline was perhaps somewhat less than that of chicks given the synthetic diet alone but the qualified significance of this difference does not permit of interpretation.

The total lipid content and the total phospholipid content of the livers of the chicks after 4 weeks is also summarized in Table I. The lipid content of the livers from chicks raised on the various diets was quite similar. In fact, a typical high-lipid "fatty liver" was not obtained inasmuch as the lipid content did not in any case exceed 7.75% (on a wet basis). Apparently sufficient choline was furnished by the yeast contained in our synthetic diet to prevent the complications of a choline deficiency. Therefore it is surprising that Topelberg and Honorato[4] using the Almquist and Klose ration low in vitamin K,[11] which also contains 7.5% yeast, state that they found a high lipid content in the livers of their unsupplemented chicks. Contrary to their report the total lipids of the livers from our chicks given vitamin K were slightly greater than the liver lipids from other chicks receiving the synthetic diet, while that of the choline-supplemented chicks was somewhat less.

The variations in total lipids were quite unrelated to the observed prothrombin times. Marked hypoprothrombinemia was typical of the chicks receiving the vitamin K-deficient diet as well as chicks consuming the same diet supplemented with choline, while the prothrombin times of the vitamin K-supplemented chicks were the same as those from chicks receiving the control natural ration. Plasma fibrinogen levels are often a sensitive indication of liver integrity.[12] The fibrinogen levels of all chicks receiving the basal synthetic diet with and without its supplements were similar.† The plasma fibrinogen of chicks on a vitamin K-free diet in which the prothrombin time was drastically prolonged resembled the fibrinogen levels in chicks receiving vitamin K.

The status of choline as a coagulant, aside from its capacity to reduce hypocoagulability resulting from failure of the liver in cases of fatty infiltration that arises in choline defi-

9 Reiner, M., *Manual of Clinical Chemistry*, 1941, New York.

10 Youngsberg, G. E., and Youngsberg, M. V., *J. Lab. Clin. Med.*, 1930, **16**, 158.

11 Almquist, H. J., and Klose, L. R., *Biochem. J.*, 1939, **33**, 1055.

12 Foster, D. P., and Whipple, G. H., *Am. J. Physiol.*, 1921-1922, **58**, 407.

† A difference in the fibrinogen levels of chicks receiving the synthetic diet in contrast to those given the natural diet indicates that plasma fibrinogen can be influenced by diet. Our observations on this subject will soon be reported elsewhere.

ciency,[13] is still obscure. For example, Bellows and Chinn[14] indicate that intraocular hemorrhages were obtained in young rats raised on a diet low in choline and methionine while Zunz and Vessoelovsky[15] did not observe any change in the clotting time of rabbits injected intravenously with choline or its homologues. In unpublished studies, it was found that choline and methionine had no effect on the hypoprothrombinemia induced in rats by 3,3′-methylenebis (4-hydroxycoumarin) and gave inconclusive results in correcting the hypocoagulability (prothrombin and fibrinogen deficiency) induced by chloroform liver intoxication in dogs maintained on an adequate dietary regime.‡

Summary. 1. The hypoprothrombinemia induced in chicks by feeding a vitamin K-free diet was not significantly influenced by the addition of choline to the diet. 2. Chicks raised on a vitamin K-free diet exhibited the typical hypoprothrombinemia but their fibrinogen levels and total liver lipids resembled those of control chicks. 3. The total lipids of the livers of chicks receiving synthetic diets were somewhat increased over the lipids from chicks given a natural ration but this condition was not corrected by vitamin K.

[13] Henry, E. M., *Biol. Symposia*, 1941, **5**, 177.

[14] Bellows, J. G., and Chinn, H., *Arch. Ophthamol.*, 1943, **30**, 105.

[15] Zunz, E., and Vessoelovsky, O., *Arch. Int. Pharmacodynamic.*, 1938, **60**, 146.

‡ Field, J. B., and Link, K. P. These observations were made during the course of studies on the anticoagulant, 3,3′-methylenebis (4-hydroxycoumarin) and will be published in the near future.

15123

Inhibition of Cytochrome Oxidase (Paraphenylendiamine Oxidase) of the Thyroid Gland by Thiouracil and Other Compounds.

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It is believed that an oxidizing enzyme plays an important role in the formation of thyroid hormone. Inasmuch as thiourea, thiouracil, sulfonamides, and other compounds have been found to suppress formation of thyroid hormone[1,2,3] it appeared of interest to study the influence of these agents on oxidizing enzymes of the thyroid gland. The experiments reported here deal with the influence of thiouracil, sulfonamides, para-aminobenzoic acid, glutathione, cysteine, ascorbic acid, and certain xanthines on the cytochrome oxidase (paraphenylendiamine oxidase) in the thyroid gland.

Methods. The cytochrome oxidase activity of thyroid tissue was determined by a modification of the method of Galli-Mainini.[4] Rats were stunned by a blow on the neck and exsanguinated by opening the chest and cutting the heart. The thyroid glands were dissected free from connective tissue, rapidly weighed on a torsion balance, and immediately dropped into a test tube containing 5 cc phosphate buffer solution pH 7 and a small amount of sand. The tissue was triturated with a glass rod. Five cubic centimeters of a freshly prepared 0.2% aqueous paraphenylendiamine solution was added. The test tubes were then shaken in a mechanical shaker for 45 minutes at 37°C. The samples were immediately filtered into a colorimeter tube and read in the Evelyn photoelectric colorimeter against a blank treated in identical manner (blank =

* J. Ewing Mears Fellow in Physiology and Medicine.

[1] Mackenzie, C. G., and Mackenzie, J. B., *Endocrinol.*, 1943, **32**, 185.

[2] Astwood, E. B., Sullivan, J., Bissel, A., and Tyslowitz, R., *Endocrinol.*, 1943, **32**, 210.

[3] Astwood, E. B., *J. Pharmacol. Exp. Therap.*, 1943, **78**, 79.

[4] Galli, Mainini C., *Rev. Soc. Argent. de biol.*, 1943, **19**, 205.

TABLE I.
Inhibition of Cytochrome Oxidase Activity (Paraphenylendiamine Oxidase) of Rat Thyroid Gland *in Vitro*. One thyroid gland of each rat served as control.

Exp. No.	No. of rats	Inhibitor	Oxidase index (per mg thyroid) Controls M	Experimental M	Difference	P of Difference*
1	11	Thiouracil 0.002 M	35.9	16.9	19.0	<0.01 S
2	8	Glutathione 0.001 M	29.7	2.7	27.0	<0.01 S
3	8	Cysteine HCl 0.001 M	42.2	38.1	4.1	>0.9
4	4	Cysteine HCl 0.002 M	28.5	15.8	12.7	<0.01 S
5	8	Na Sulfadiazine 0.001 M Na Sulfathiazol 0.001 M	35.8	26.8	9.0	<0.01 S
6	8	Na Sulfadiazine 0.0005 M Na Sulfathiazol 0. 0005 M	33.3	21.5	11.8	<0.05 S
7	18	Na Sulfathiazol 0.001 M	23.1	17.7	5.4	<0.01 S
8	14	Na Sulfadiazine 0.001 M	21.0	17.9	3.1	<0.4
9	14	Paraaminobenzoic Acid 0.001-0.003 M	28.6	30.5	insignificant increase	<0.5
10	4	Ascorbic Acid 0.001 M	25.5	0	complete inhibition	
11	2	Paraxanthin 25 mg %	32.4	36.7	insignificant increase	<0.1

* P values calculated according to "Student's t-method," R. A. Fisher: *Statistical Methods for Research Workers*, 1932.
Significant values are marked in the table by "S."

TABLE II.
Oxidase Index of Thyroids of Rats Treated with Thiouracil for 11 to 21 Days, as Compared with Normal Controls.

Normal controls No. of animals	Normal controls Oxidase index* mean	Thiouracil treated No. of animals	Thiouracil treated Oxidase index* mean	Diff. of indices	P of Diff.†
55	28.9	25	21.6	7.3	<0.01 S
	Corr.‡ 49.8		Corr.‡ 22.9	26.9	

* Oxidase index calculated per mg thyroid weight.
† See footnote *, Table I.
‡ Index corrected to give oxidase activity per mg epithelial weight. See text.

100) using filter 635. An index of enzyme activity per mg thyroid was calculated as follows:

$$I = \frac{2 - \log G}{(\text{weight in mg})}$$

In one series of experiments the influence of thiouracil,† sodium sulfathiazole, sodium sulfadiazine, glutathione, cysteine hydrochloride, paraaminobenzoic acid, ascorbic acid, and xanthin‡ compounds was studied *in vitro*. These were dissolved in the buffer solution, prior to the addition of the thyroid tissue, in amounts to give final concentrations as indicated in Table I. Blanks were treated in the same manner. One thyroid gland of each animal was used with the test material, the other serving as a control. A blank containing no inhibitor was used for the control glands.

In a second group of experiments the oxidase activity of the thyroid glands of rats treated with thiouracil was compared with that of normal control rats. Adult male and female rats were used, maintained on a stock diet of Purina dog chow, with lettuce added twice weekly. The experimental animals received thiouracil in 0.05% solution as drinking solution for a period of 11-21 days.

† Thiouracil was supplied by the Lederle Laboratories through the courtesy of Dr. Stanton M. Hardy.

‡ Paraxanthin was kindly prepared for us by Dr. D. Turner, Jefferson Medical College, Philadelphia.

Results. Results of *in vitro* experiments are summarized in Table I. Thiouracil, sodium sulfathiazole and a mixture of sodium sulfathiazole and sodium sulfadiazine inhibited oxidase activity significantly. Ascorbic acid produced complete, and glutathione almost complete inhibition; in 0.001 M solution both were much more potent inhibitors *in vitro* than were the antithyroid drugs. It is interesting that cysteine hydrochloride, the reducing power of which is similar to that of glutathione, did not inhibit in equimolecular solution (0.001 M). However, it did inhibit in 0.002 M solution, its inhibiting action in this concentration being of the same magnitude as that of 0.002 M thiouracil solution.

Paraaminobenzoic acid and paraxanthin failed to inhibit oxidase activity in the concentrations employed. Not included in the table are several experiments with other xanthin derivatives, theophyllin and theobromine as well as a mixture of both, which also failed to inhibit the oxidase.

The *in vivo* experiments are summarized in Table II. Adult animals were used for controls and treated. Inasmuch as there was no apparent difference between males and females and the results obtained with treatment periods of 11-21 days were uniform, all values for this period were pooled. The cytochrome oxidase activity of the thyroids of the treated animals were significantly lower than that of the controls. Data on rats treated for shorter or considerably longer periods are not given in the table since these data are incomplete. It does appear, however, that after treatment for many months the oxidase activity of the thyroid glands increases, approaching normal. In these as in all experiments, the oxidase index of the thyroid was calculated per unit of weight. It may be assumed that the enzyme is present largely in the cells rather than the colloid. Therefore, comparison of the enzyme activity per weight unit of normal control glands rich in colloid, and with low epithelium with that of glands of thiouracil-treated animals, devoid of colloid and with hyperplastic epithelium may be misleading. Since in most experimental and control animals both glands had been used for the enzyme studies correction could not be made for each thyroid gland. However, representative sections of thyroids of normal and of thiouracil-treated rats were drawn on paper by means of a projector; colloid and interstitial tissue areas were cut out and the epithelial area weighed. The ratio of total area weight to epithelial area weight was used to calculate the oxidase index per mg epithelial weight instead of per mg thyroid weight. These figures, admittedly representing only a gross approximation, are presented in Table II as "corrected indices." This correction emphasizes the diminution in cytochrome oxidase activity in the treated glands, which was apparent even when calculated on the basis of weight of the entire gland.

Discussion. Of the compounds studied by us, thiouracil and sulfonamides are known to suppress formation of thyroid hormone[1,2] and to decrease the uptake of iodine by the thyroid gland of treated subjects.[5-9] Thiouracil[10] and sulfonamides[11] inhibit the formation of diiodotyrosin and thyroxin but not the uptake of inorganic iodine, by thyroid slices *in vitro*. The mechanism of formation of diiodotyrosin and thyroxin is not fully understood but there is evidence that an oxidative enzymatic mechanism is involved, possibly in the freeing of I from inorganic iodide.[12] Dempsey[13] showed that the histological peroxidase reaction of thyroid tissue can be inhibited by immersing sections in thiourea or thiouracil solutions. However, he found no difference between the histological peroxidase reaction in thyroids

5 Bissel, A., and Astwood, E. B., *Endocrinol.*, 1944, **34**, 282.

6 Baumann, E. J., Metzger, N., and Marine, D., *Endocrinol.*, 1944, **34**, 44.

7 Rawson, R. W., Tannheimer, J. F., and Peacock, W., *Endocrinol.*, 1944, **34**, 245.

8 Keston, A. S., Goldsmith, E. D., Gordon, A. S., and Charipper, H., *J. Biol. Chem.*, 1944, **152**, 241.

9 Rawson, R. W., Evans, R. D., Means, J. H., Peacock, W. C., Lerman, J., and Cortell, R. E., *J. Clin. Endocr.*, 1944, **4**, 1.

10 Franklin, A. L., Chaikoff, J. L., and Lerner, S. R., *J. Biol. Chem.*, 1944, **153**, 151.

11 Franklin, A. L., and Chaikoff, J. L., *J. Biol. Chem.*, 1944, **152**, 295.

12 Schachner, H., Franklin, A. L., and Chaikoff, J. L., *J. Biol. Chem.*, 1943, **151**, 191.

13 Dempsey, E. W., *Endocrinol.*, 1944, **34**, 27.

from thiouracil-treated rats and normal controls. It has been suggested on theoretical grounds that the formation of thyroxin from diiodotyrosin may be dependent on peroxidase action.[14] However, Glock[15] has shown that the thyroid gland (of the horse) contains no peroxidase.

Polyphenol oxidase (tyrosinase) is inhibited *in vitro* by phenylthiourea,[16] sulfonamides,[17] and by thiouracil, and a number of other compounds[18] but since this enzyme is not present in thyroid tissue these observations merely indicate that thiourea and thiouracil inhibit various oxidizing enzymes but throw no light on the mechanism of inhibition of thyroid hormone formation. Schachner *et al.*[12] have found that *in vitro* formation of thyroxin and diiodotyrosin in thyroid tissue slices is inhibited by KCN, H_2S, CO, and NaN_3. Only two oxidizing enzymes are known to be inhibited by all of these inhibitors, *i.e.*, cytochrome oxidase and polyphenol oxidase. Because of the absence of polyphenol oxidase in thyroid tissue and because of the type of inhibition produced by CO in their experiments, these authors concluded that cytochrome oxidase is the oxidizing enzyme involved in the formation of thyroid hormone.

Our data, a preliminary report of which has appeared previously,[19] indicate that two groups of antithyroid drugs, thiouracil and sulfonamides, which are known to inhibit formation of thyroid hormone, actually inhibit the cytochrome oxidase (paraphenylendiamine oxidase) activity of the thyroid gland *in vitro*. The oxidase activity of thyroid glands of animals treated with thiouracil is likewise decreased. Since there is suggestive evidence that the cytochrome oxidase system is involved in the formation of thyroid hormone[12] and since we have found that this enzyme system is inhibited by thiouracil and sulfonamides, it appears possible that inhibition of this enzyme may constitute the basis for the antithyroid effect of these agents.

It should be noted that the experimental procedure employed by us reflects upon the entire cytochrome oxidase system, no attempt having been made to isolate either cytochrome or cytochrome oxidase, both of which are involved in the oxidation of paraphenylendiamine.

A number of compounds were studied in addition to thiouracil and sulfonamides. Of these, paraminobenzoic acid is of interest because Astwood[3] found that it induced moderate thyroid hyperplasis and because of recent claims[20] that it is useful therapeutically in thyrotoxicosis. The agent had no inhibitory effect on the cytochrome oxidase activity of the thyroid in concentrations employed by us, in striking contradistinction to its inhibitory effect on potato tyrosinase.[18] The effect of paraxanthin was investigated, with negative results, because of the report of Carter *et al.*[21] Glutathione, and ascorbic acid, and to a lesser extent cysteine hydrochloride, proved to be potent inhibitors *in vitro*. Whether this fact has any physiological significance is questionable. Astwood obtained no antithyroid effect in rats with ascorbic acid[3] whereas Heyl[22] reported morphological effects in guinea pigs similar to those subsequently obtained with antithyroid drugs. Our own observations[23] in guinea pigs have failed to show significant hyperplasia after administration of ascorbic acid.

Summary and Conclusions. 1. Thiouracil and sulfonamides inhibit the cytochrome oxidase activity (paraphenylendiamine oxidase) of thyroid tissue (rat) *in vitro*. 2. The cytochrome oxidase activity of thyroid glands of rats treated with thiouracil is subnormal. 3. Inhibition of cytochrome oxidase activity

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17 Martin, G. J., Wisansky, W. A., and Ansbacher, S., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 26.

18 Paschkis, K. E., Cantarow, A., Hart, W. M., and Rakoff, A. E., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 37.

19 Paschkis, K. E., Cantarow, A., Rakoff, A. E., and Tillson, E. K., *Fed. Proc.*, 1945, **4**, 55.

20 Berman, L., PROC. SOC. EXP. BIOL. AND MED., 1945, **59**, 70.

21 Carter, G. S., Jenkins, G. N., Mann, F. G., and Harley-Mason, J., *Nature*, 1943, **151**, 728.

22 Heyl, J. G., *Acta brev. Neerland*, 1934, **4**, 12.

23 Paschkis, K. E., unpublished observations.

may possibly be the factor responsible for the antithyroid activity of these drugs. 4. Glutathione, cysteine hydrochloride, and ascorbic acid inhibit cytochrome oxidase activity (paraphenylendiamine oxidase) of thyroid tissue *in vitro*. 5. Paraminobenzoic acid and paraxanthin do not influence the cytochrome oxidase activity of the thyroid gland.

15124

Antibody Response of Swine to Repeated Vaccination with Formalin-Inactivated, Purified Swine Influenza Virus.*

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The results of studies on the relation of antibody response of swine to dose of purified swine influenza virus treated with formalin have been described in a recent report.[1] Within the range of dosage employed, 0.1 to 5.0 mg of formolized virus per 100 lb of animal weight, the relation of log antibody titer to log dose was essentially linear. In a single vaccination, a 10-fold difference in dose was associated with a 2.5-fold difference in titer. The maximum titer of serum taken at weekly intervals occurred in the serum obtained 1 week after vaccination; within 3 weeks after vaccination the average titers associated with all doses had declined to similar levels, which were about three 2-fold dilutions below the maximum average titer of the animals receiving the largest doses.

Because of the relatively small influence of the magnitude of the dose and the surprisingly rapid decline in titer, a second vaccination was made 6 weeks after the first. The relation of log antibody response to log dose was again apparently linear and a 10-fold difference in dose was associated with a 1.6-fold difference in titer. In contrast with the results of the first vaccination, however, the maximum titer, which occurred 7 to 9 days after the second vaccination, greatly exceeded that following the single vaccination. For comparable doses per unit weight, the antibody titer after the second vaccination rose to a level approximately three 2-fold dilutions above that seen after the first vaccination. Furthermore, the titers observed 6 weeks after the second vaccination, except those resulting from the smallest doses of vaccine, had not sunk to the level reached 3 weeks after the initial vaccination.

These and other results demonstrated that the degree of antibody response was far more dependent on the status of the host with respect to previous experience with the influenza virus or its derivatives than on the mass of formolized virus introduced at vaccination. In this respect it was observed that the maximum response to the dose of 0.125 mg per 100 lb at the second vaccination was approximately twice that to the dose of 2.0 mg given in the first vaccination. Inasmuch as the cost

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1 McLean, I. W., Jr., Beard, D., Taylor, A. R., Sharp, D. G., and Beard, J. W., *J. Immunol.*, 1945, **51**, 65.

and the difficulty of preparing vaccines of purified and concentrated influenza virus increase rapidly with the size of the dose, it is possible that repeated vaccination with small doses of vaccine would be more advantageous and desirable in obtaining a high antibody response than the use of a single large dose. Limitation of the dose is especially desirable in consideration of general reactions which have been observed both in men and swine[1] when the dose is increased beyond certain levels.

In the previous study repetition of vaccination was carried out at the period of 6 weeks after the initial administration of vaccine. The 6 weeks interval was employed because it was only after this period that the course of antibody response to the first vaccination was apparent. In order to learn more of the influence of the sequence of vaccination with respect to the interval between vaccinations, further study of the problem was undertaken. An investigation was made of the response of swine to 2 vaccinations spaced at intervals of 1 to 4 weeks, and the results obtained are described in the present report.

Materials and Methods. The virus used for the preparation of the vaccine was the same strain of egg-adapted swine influenza virus obtained from Dr. John F. Enders that was employed for the chemical, physical, biological, and immunological studies previously reported.[1-4]

Concentration of the virus and preparation of the vaccine. The vaccine employed in the present experiments was from a stock different from that used in the previous work.[1] Propagation and harvest of the virus was carried out as before. Concentration of the virus was accomplished with the modified[5] Sharples centrifuge in a manner similar to that previously described,[1] except that use was made of ultraviolet light for maintenance of aseptic conditions during centrifugation. The whole technic for getting the virus and concentrating it was essentially identical with that employed[5] in concentrating influenza viruses A and B for the preparation of vaccines. The virus from 16,100 ml of bacteriologically sterile chorio-allantoic fluid (yield from 1,700 eggs) was concentrated in 100 ml of sterile Ringer's solution, with a loss of only 9.5% of the starting hemagglutinative activity. Electron micrographs and sedimentation velocity studies of this preparation gave results indistinguishable from those previously reported[4] for this virus. The specific infectivity for chick embryos was $10^{-13.5}$ g of virus, and the specific hemagglutinative activity was $10^{-6.95}$ g of virus (based on a nitrogen factor[3] of 11.1). Cultures of the concentrate in beef extract broth and glucose agar showed no contaminating bacteria to be present. Anaerobic cultures were likewise negative.

To 86 ml of the concentrate containing 4.44 mg of virus per ml there was added sterile Ringer's solution containing 19.1 ml of 0.5% formalin and 1.91 ml of 1/500 solution of phenyl mercuric borate, to give a total volume of 191 ml of vaccine containing 0.05% formalin, 1/50,000 phenyl mercuric borate and 2.0 mg of virus per ml. After standing for 7 days at room temperature (28 to 30°C), the vaccine did not contain enough active virus to infect chick embryos. Cultures of the vaccine showed no viable bacteria to be present.

The vaccine was stored at the concentration of 2.0 mg per ml in a tightly stoppered bottle at 2-8°C for 4 months before final dilution for use. At the beginning of the present work 34 ml of the vaccine were diluted with 102 ml of sterile Ringer's solution containing 0.05% formalin and 1/50,000 phenyl mercuric borate to yield 136 ml of vaccine containing 0.5 mg of formolized virus per ml. This was partitioned in 17-ml volumes in vaccine bottles and stored at 2-8°C until used. An electron micrograph of the vaccine taken 11 months after preparation is shown in Fig. 1.

The experimental animals were swine obtained in Orange County, North Carolina. At the time of the first vaccination, the group

2 Taylor, A. R., Sharp, D. G., McLean, I. W., Jr., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1944, **48**, 361.

3 Taylor, A. R., *J. Biol. Chem.*, 1944, **153**, 675.

4 Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, D., and Beard, J. W., *J. Biol. Chem.*, 1944, **156**, 585.

5 Taylor, A. R., Sharp, D. G., McLean, I. W., Jr., Beard, D., and Beard, J. W., *J. Immunol.*, 1945, **50**, 291.

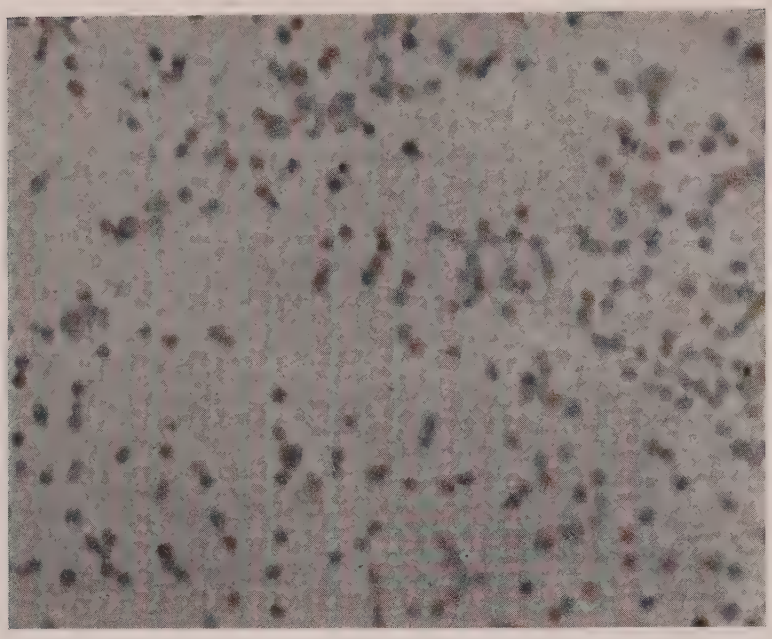

FIG. 1.

Swine influenza virus vaccine stored at 2-8°C for 11 months. The electron micrograph was taken at 55 kilovolts, and the total magnification is 19,700 ×. Comparison of this micrograph with that of 2, Fig. 2 reveals no essential difference between the appearance of the images of the formolized virus particles stored for this period and the images of the untreated, fully active particles immediately after purification.

of 64 varied in age from 16 to 20 weeks and weighed from 42 to 94 lb., with an average of 60.8 lb. There were 30 females and 34 males; 31 of the males had been castrated. The animals were predominently of Berkshire strain, with some admixture of Poland China, Hampshire, Duroc, and O.I.C.

The animals were divided into 4 groups of 16 each so that each subgroup was representative of the whole with respect to weight and sex. The average individual weights for the 4 groups, A, B, C, and D, were 59.1, 61.0, 61.7, and 61.4 lb, respectively. The pigs were kept on open range with sheds to provide protection from the rain and were fed from self feeders with a commercial balanced feed. Weight gain was satisfactory, being from 4.5 to 9 lb per week, and all of the animals appeared healthy throughout the experiment.

Vaccination was performed by introducing 1 ml of the vaccine into the subcutaneous tissue of the right posterior axillary region. No local or general reaction was observed in any instance. Weights were taken at the time of each vaccination and at the termination of the experiment, 6 weeks after the second injection. Blood was drawn from the femoral vein or artery immediately before each vaccination and 1, 2, 4, and 6 weeks after vaccination. The blood samples were left for about 18 hours at room temperature to allow for retraction of the clot, and the sera were then separated from the clots and centrifuged to remove suspended red blood cells. A sample of each serum was set aside for titration of antibody content before freezing and the remainder divided into 2 portions in individual vials, frozen, and stored at −10°C.

Serum antibody levels were estimated by a modification of the hemagglutinin-inhibition method of Hirst, Rickard, Whitman, and Horsfall[6] previously described.[1] It was found during the course of the work that while duplicate titrations set up at the same time with the same materials showed almost exact reproduction of endpoints, the results of titra-

[6] Hirst, G. K., Rickard, E. R., Whitman, L., and Horsfall, F. L., *J. Exp. Med.*, 1942, **75**, 495.

TABLE I.
Dates of Vaccination and Bleeding.

Animal group	September						October										November			
	11	12	18	19	25	26	2	3	9	10	16	17	23	24	30	31	6	7	13	21
A	BV*		BV		B		B				B				B					
B	BV		B		BV		B		B				B				B			
C		BV		B		B		BV		B		B				B			B	
D		BV		B		B				BV		B		B				B		B

* B = Bled; V = Vaccinated.

tions of the same sera at different times using different preparations of cells and other materials exhibited a standard deviation of 0.6 of a 2-fold dilution interval, or 30% variation. In order that an overall comparison of results could be made, each serum was titrated in triplicate: (1) before it was frozen and within 4 days of the bleeding time (see Table I); (2) after storage in the frozen state until the completion of the experiment with a given group; and (3) after storage in the frozen state until the termination of the entire experiment. Freezing and storing made no significant difference in the level of antibody titer obtained by the hemagglutinin-inhibition method.

The titers referred to in this paper are the geometric means of the 3 titration endpoints observed. Common logarithms have been used for expression of the titers and for calculations throughout, rather than the arithmetic titers. The arithmetic means, being unduly influenced by the values of serums of very high titer, do not as closely approximate the medians and the modes of the groups as do the geometric means.

Results. The 4 groups of animals were given the first dose of vaccine at essentially the same time as shown in Table I. Subsequent vaccination was performed, Table I, at 1, 2, 3, and 4-week intervals for Groups A, B, C, and D, respectively. The sequence of bleeding in relation to the vaccination is likewise shown in Table I.

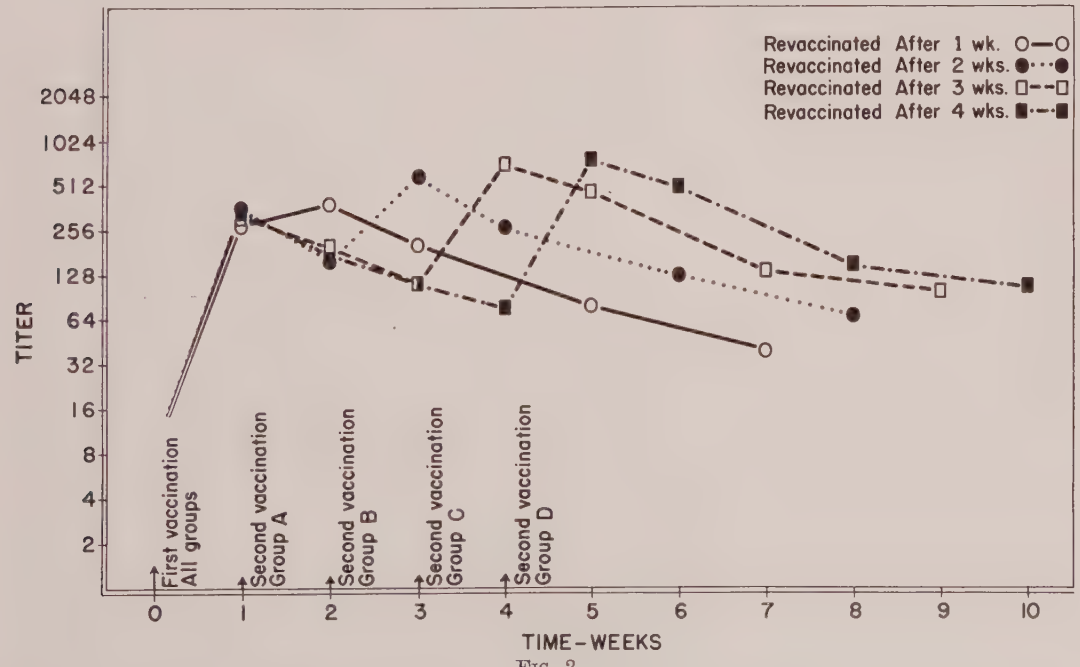

FIG. 2.

The antibody response to a single vaccination and to repeated vaccination at various intervals after the first.

The overall results of the experiment in terms of average group antibody response in relation to the sequence of vaccination are given in Fig. 2. The average antibody responses of the 4 groups of animals to the first vaccination were uniform, and the peak responses were observed in the serums taken 1 week after vaccination in the three groups, B, C, and D, which were not revaccinated at the 1-week interval. The similarity of the responses of the various groups to the single vaccination is seen in Fig. 2 and further demonstrated by the agreement—better than 0.3 of a 2-fold dilution interval—obtained between the mean log titers of the respective groups following one injection of vaccine.

In the present experiments, as in those previously described ([1], Fig. 4), the range of antibody titers of the serums of individual animals within the respective groups at a given bleeding was large, varying from about 2 to 4 2-fold dilution intervals, with an overall standard deviation from the means within groups of 1.48 2-fold dilutions. Analysis of the data of the triplicate titrations on each serum as described above showed a standard deviation of 0.56 of a 2-fold dilution interval which was attributable chiefly to variations in the technic of titration. From these two values, the standard deviation of the antibody titers of the serums within individual groups was estimated to be 0.8 of a 2-fold dilution interval. This variation is that related to differences in host response.

As noted above, the variation in the average antibody responses of the respective groups to the first vaccination was less than 0.3 of a 2-fold dilution. This value represents a variation which is less than would be expected from chance alone. The average of the mean responses of the entire group of 64 animals was taken as the best available estimate of the response to the single vaccination, and analyses of the effects of the second vaccination were made with this value as a basis.

Examination of the findings subsequent to the 1-week interval after the first vaccination reveals (1) a rapid, progressive decline in the average antibody titers of the respective groups of animals in the intervals between the first and second vaccinations; (2) the attainment of progressively higher maximum antibody *levels* in relation to the length of the interval between vaccinations; (3) the formation of progressively greater *amounts* of antibody in relation to the length of the interval between vaccinations; and (4) the tendency toward maintenance of higher antibody levels for longer periods in those animals held for the longer intervals between vaccinations.

The rapid decline in antibody titer following the initial vaccination in the present experiments was closely similar to analogous findings in the work already described.[1] The uniformity in the rate of decline in the average antibody levels of respective groups is evident.

The second vaccination resulted in all instances in an increase in the *average* antibody level of the respective groups above the maximum level reached after the first vaccination. The maximum level attained by each group after revaccination was observed, within the limits of the intervals in which bleedings were made, at the period of 1 week after the second vaccination. It is seen in Fig. 2, however, that the height of the maximum titer reached after the second vaccination was influenced by the interval elapsing between the two vaccinations, so that with increase in this interval a progressively higher average maximum titer was attained by the respective groups. Examination was made of the statistical significance of these progressively higher maximum antibody levels. Analysis of variance[7] of the pooled data of the entire experiment including the findings resulting from both first and second vaccinations indicated the least significant difference between group mean log titers ($t_{0.05}\ \bar{sd}$) to be 0.18 or 0.59 of a 2-fold dilution interval, a value which is much greater than the differences observed after the first vaccination. The average log antibody titer of the whole group of 64 animals 7 days after the first vaccination was 2.50 and the average log titers of the groups A, B, C, and D 7 days after the second injection were 2.57, 2.74, 2.83, and 2.86, respectively. The increases in the maximum mean log antibody titers of groups A, B, C, and D with revaccination above the mean log

[7] Snedecor, G. W., *Statistical Methods*, Ames, The Iowa State College Press, 3rd edition, 1940.

antibody titer of the whole group of 64 animals after a single vaccination thus were 0.07, 0.24, 0.33, and 0.36. From these values it is seen that differences between the increases in the titers of the adjacent groups, that is, between those of A and B or B and C, are too small to be of unequivocal significance. However, when the increase in log titer of Group D, 0.36, is compared with that of Group A, 0.07, the difference is seen to be a full 2-fold dilution, a difference which is highly significant. This finding, together with the trend shown in Fig. 2, provides strong evidence of the existence within the limits of the duration of the experiment of a direct relation between the maximum titer attained and the length of the interval separating the two vaccinations.

The dependence of the total average *amounts* of antibody formed by the different groups on the interval between vaccination is evident from inspection of Fig. 2. This finding is in accord with the observation in the previous work of the influence on response of prior experience of the host with the influenza virus or vaccine. The differences in the amounts of antibody formed by the respective groups in the present work were great. None of the animals used in the study showed any evidence of previous exposure to the swine influenza virus, since all of the titers before any vaccination were less than 16. Assuming that none of the serums contained any antibody before the first vaccination, the average *amount* of antibody formed in the whole group of 64 pigs as the result of the first vaccination was 315 units. The increases in antibody units of the various groups above the level present at the moment of second vaccination were 100, 400, 570, and 645 units for Groups A, B, C, and D, respectively. These amounts correspond to increases of 53, 223, 360, and 406 units above the average amount, 315 units, present one week after the first vaccination.

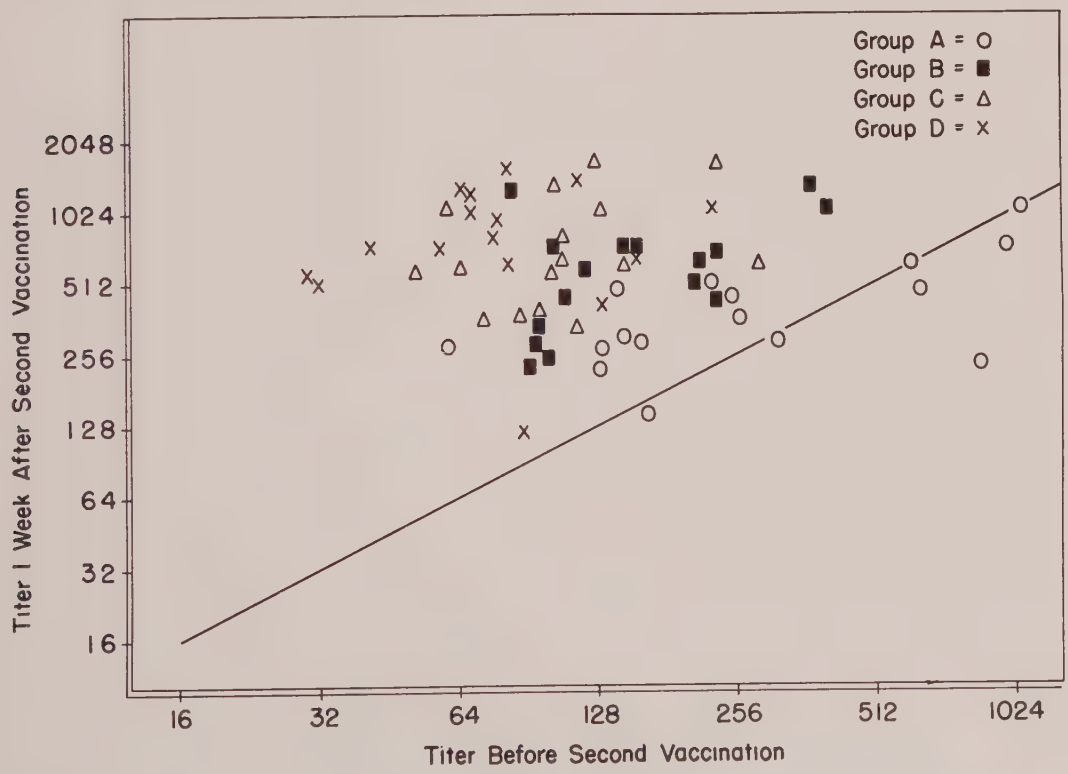

FIG. 3.

Individual antibody responses 7 days after second vaccination compared with the antibody titers immediately before second vaccination. The continuous line represents the base line of the antibody titers of the serums obtained immediately before the second vaccination.

The rate of decline in the average antibody level after the second vaccination was not greatly different in the various groups. As a consequence the residual titers of those groups having the higher titers following the second vaccination remained at higher levels during the later stages of the experiment. It appeared possible that the decline in Groups C and D was slower toward the end than that in Groups A and B, but the data were insufficient to provide an answer to this question.

The responses of the individual animals of the various groups to the second vaccination are shown in the spot chart of Fig. 3. Here the titers observed 7 days after the second vaccination are compared with the antibody titers of the individuals immediately before the second vaccination. The results given in Fig. 3, like those in Fig. 2, show that, though individual variation was great, quantitative response to second vaccination was greatly dependent on the time relations of previous experience of the animals with the vaccine. The animals with the lowest titers were those revaccinated after the longest period following first vaccination, and it was in these pigs that the largest amounts of antibodies were formed in response to the second vaccination. The poorest individual responses were seen in the animals of Group A which had the highest titers before second vaccination. As shown in Fig. 3, seven of the animals of this group either lost or experienced no gain in antibody titer after the second vaccination, while all of the animals of the other groups gained in antibody titer following revaccination.

Discussion. Previous studies on the vaccination of swine with formalin-inactivated swine influenza virus have shown that the magnitude of antibody response to a single vaccination is affected in relatively small degree by the size of the dose given. Similar relations were again apparent when vaccination was repeated in the same animals after a period of about 6 weeks from the time of the initial vaccination. In striking contrast to these results was the observation that the response to second vaccination with a given dose was much greater than that to the first. It was thus clear that the primary factors concerned with the height of antibody response resided in the host and that attempts to obtain maximum response should be directed more toward conditioning or preparation of the host, as by repeated vaccination, than toward manipulation of the dose of vaccine administered.

The results of the present work have shown that response is conditioned not only by the character of the stimulus but by the manner in which it is applied. As before, maximum response was observed after second vaccination. The magnitude of response, however, was found to be largely dependent on the interval elapsing between vaccinations. Within the limits of the period of study, the greater the interval before repetition of vaccination, the higher were the levels of antibody titer attained and the larger were the absolute amounts of antibody formed. It was observed, likewise, that a similar relation existed for the height of residual antibody titer at the 6-weeks period following revaccination, a relation due in large part to the similarities of the rates of antibody loss, which were relatively independent of the height of titer. The optimum interval between vaccination, from the point of view of the attainment and maintenance of the highest antibody levels in swine, would appear to be about 3 weeks. Choice of the proper interval is dependent on consideration both of the titer declining rapidly after the first vaccination and the magnitude of response to be achieved after the second vaccination.

Certain of these findings, in so far as a comparison can be made, are apparently contrary to those which Hirst, Rickard, Whitman, and Horsfall encountered in studies on the vaccination of man. From a study of the serums from 400 people, these authors concluded that "the amount of antibody produced by a group with a low prevaccination antibody level was very nearly the same as the amount produced by groups that had higher initial levels." In the present work this was by no means the case, for those animals having the lowest antibody titers before the second vaccination produced much greater amounts of antibody than those with the highest levels. The relation observed here, therefore, was concerned not with actual titer but with the immunological

status of the host. It should be emphasized, however, that the results in swine were related wholly to effects of formolized virus, whereas the findings in man were related to the effects of possible actual infection with active virus followed by vaccination. Further, the group of swine studied had been treated under controlled conditions in contrast with the people who were chosen at random. For example, in the present work the animals with the highest titer before second vaccination were those revaccinated after the shortest interval between vaccination when conditions for response were not optimum; in the group of people, those with the highest titers may have represented either the best reactors or those recently recovered from the disease.

The results of the experiments with swine were similar to those of Hirst, Rickard, Whitman and Horsfall[6] in indicating rapid loss in antibody titer subsequent to vaccination, regardless of the sequence of vaccination.

Summary. The antibody response of swine to second vaccination with formalin-inactivated swine influenza virus was greatly influenced by the period between vaccinations. Second vaccination at intervals of one to four weeks resulted in antibody titers and amounts progressively greater in direct relation to the length of the interval. In parallel higher antibody levels were maintained for longer periods in those animals receiving the second vaccination after the longer intervals. Considering the height of titer after the second vaccination, together with the rapid loss of antibody after the first injection, the interval between vaccinations optimum for maintenance of the highest antibody levels appeared to be about three weeks.

15125

Absorption of Penicillin from the Nose and Alimentary Canal.

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(With the technical assistance of Doris W. Jenkins and Mary W. Cutting.)

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To obviate the necessity for parenteral injection of penicillin other routes have been sought. Administration by mouth, including enteric capsule and duodenal tube, and by rectum have been tried as summarized previously.[1] Although satisfactory absorption results from several such combinations or procedures, the efficiency falls short of that from injection of the drug.

In this report, the relative absorption by various routes in man and animals are compared.

Absorption from the Alimentary Canal of Rats. Under sodium pentobarbital anesthesia (35 mg per kg intraperitoneally), rats were given penicillin intramuscularly and in ligated sections of the alimentary tract as follows: esophagus, stomach, duodenum (3 cm), ileum (3 cm), colon (3 cm). Ten animals were used for each route, with doses of penicillin varying from 500 to 12,500 u. per kg, body weight. Blood was taken from the heart 30 minutes later for penicillin assay. Assays were made by the method of Wolohan and Cutting[2] on whole blood. The average results, in units per cc of blood, were as follows: esophagus 0.08, stomach 0.02, duodenum 0.02, ileum 0.03, colon 0.02, intramuscular 0.11.

It is evident that absorption in the rat occurs from the stomach, duodenum, ileum, and colon about equally, but only about one

1 Cutting, W. C., Halpern, R. M., Sultan, E. H., and Armstrong, C. D., *J. A. M. A.*, 1945, **129**, 425.

2 Wolohan, M. B., and Cutting, W. C., *J. Lab. and Clin. Med.*, 1945, **30**, 161.

TABLE I.
Average Blood Concentration of Penicillin after Intramuscular, Intranasal, and Oral Administration.

	15 min	1 hr	2 hrs	4 hrs
Intramuscular	—	0.19	0.16	0.08
Intranasal	0.05	0.04	0.03	0
Oral	0.03	0.04	0.03	0.03

fifth as well as from intramuscular injection. The surprisingly high concentrations after esophageal administration are interesting, but probably of no practical value.[1]

Oral and Nasal Absorption in Dogs. Penicillin was given to 8 dogs: 2 intramuscularly, 3 intranasally (pentobarbital anesthesia, 35 mg per kg intraperitoneally), and 3 orally (esophagus ligated, same anesthesia). The dose in each instance was 1000 U. per kg, body weight, in physiological salt solution containing 100,000 U. per cc. Blood was obtained at approximately 15 minutes, and 1, 2, and 4 hours, for penicillin estimations. The average results (Table I) indicate a similar low absorption from the mouth and nose, and several fold higher blood levels after intramuscular injection.

Effect on Ciliary Activity in Frogs. The effect of penicillin on ciliary activity was studied because of the possible use of ciliated membranes (nose) as absorptive areas.

Isolated frog esophagus was split longitudinally into halves, laid flat on a cork-board, and moistened with Ringer's solution. The reciprocal of the average time (of three readings) for a small granule of cork to travel 1 cm distance was taken as the initial activity. Next a cotton pledget soaked with Ringer's solution was applied to one-half of the esophagus (control) and a pledget soaked in penicillin in Ringer's solution to the other half, each for 5 minutes. Then the ciliary activity was re-determined in the same way. The percents of ciliary activity in the penicillin-treated strips, as compared with the controls, were +110%, +53%, and +21% of the initial values for penicillin concentrations of 12,500, 25,000, 50,000 U. per cc respectively. Concentrations of 100,000 U. per cc caused complete stoppage of ciliary activity, which recovered gradually after 30 minutes. The results are presented in Fig. 1.

In general, it appears that penicillin, in low concentrations, causes a slight, though fleeting, stimulant effect on ciliary activity, while higher concentrations (more than 25,000 U. per cc) cause depression, and finally stoppage with 100,000 U. per cc. However, the depression is fairly readily reversible on standing (½ to 2 hours) with washing.

Intranasal Absorption of Penicillin in Man.

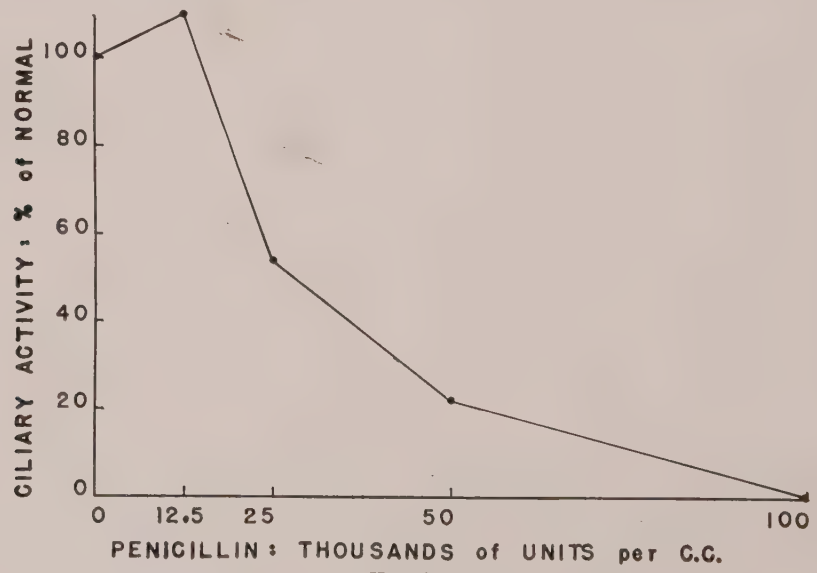

FIG. 1.
The effect of penicillin on ciliary activity in frog esophagus.

The nose was tested as a site for the absorption of penicillin in man. Preliminary trials with solutions of the agent in physiological salt solution dropped into the nose showed that concentrated solutions (100,000 U. per cc) were too irritating for general use. Intense burning, lasting for 10 seconds to a minute, was produced, and could not be circumvented by incorporating 15% saligenin or 1% tetracaine. The use of cottonseed oil, 5% dextrose, or 50% carbowax 1200 likewise did not prevent a burning sensation.

When penicillin was sprayed into the nose from a clinical hand atomizer, however, little or no irritation was experienced with solutions containing 100,000 units per cc. In several persons, repeated administrations, at 2- or 3-hour intervals, were given, without the production of disagreeable symptoms. One-fourth cc was well retained in the nose, while 0.5 cc overflowed into the posterior pharynx. The penicillin was usually placed in a short (about 3 cm) test tube, which was placed inside the bowl of the atomizer, and into which the exhausting tube dipped.

The concentration of penicillin in the blood after the administration of 25,000 units in 0.25 cc of physiological salt solution by nose was estimated at 30-minute to 1-hour intervals for 2 or 3 hours in 29 trials in 10 normal persons, and in 12 patients without upper respiratory disease.

As many bloods contain an appreciable amount of antistreptococcal activity,[3] which would enhance the activity of any penicillin present, control assays were made on initial samples of blood, before the penicillin was administered, and any indicated activity was subtracted from subsequent estimations. From the 29 trials, a total of 94 blood estimations were obtained, with an average penicillin concentration of 0.02 U. per cc. The highest concentration obtained was 0.06 U. per cc (9 trials), while in 7 trials no absorption could be demonstrated. Within the 2 to 3 hours of the test variations from 0 to 0.06 U. per cc might occur, but the average concentration at the end of the trial period (0.02 U. per cc) was the same as the average of the initial readings, showing a rather steady absorption, in contrast to that by other routes.

Since a blood concentration of 0.02 U. per cc cannot be considered adequate for treatment of most systemic infections, the intranasal route is not of promising value in this direction. However, the combination of a low blood and tissue concentration, together with a high local concentration in the nose, may be of value in the treatment of upper respiratory infections of bacterial origin. At present, trials are in progress in acute colds (following the initial and presumably virus stage) and sinusitis.

Discussion. In an attempt to avoid parenteral injection, the absorption of penicillin from a number of other sites was studied. Absorption can be demonstrated from the nose, and from practically any part of the alimentary tract, in animals. The sites of greatest promise appear to be the upper intestine and the nose. The latter organ has been inadequately tried in this connection, and although resulting systemic concentrations are low, and somewhat variable, they may not be without merit in upper respiratory tract infections, in connection with the high local concentration simultaneously produced.

Conclusions. 1. Penicillin is absorbed from the esophagus, stomach, duodenum, ileum, or colon of rats about one-fifth as well as from intramuscular injection. 2. Penicillin is also absorbed from the nose or mouth of dogs about one-fourth as well as from intramuscular injection. 3. Concentrations higher than 25,000 units of penicillin per cc depress, reversibly, the ciliary activity in frog's esophagus. 4. Penicillin may be sprayed into the nose in concentrations of 100,000 units per cc without producing significant irritation. 5. Although the blood concentrations following intranasal spraying of penicillin are low, they may enhance local antibacterial effects in the upper respiratory tract.

[3] Elias, W. F., Merrion, H. J., and Speicher, T., *Science*, 1945, **102**, 223.

15126 P

Regulatory Effect of Adrenal Cortical Extract on Elaboration of Pituitary Adrenotropic Hormone.*

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Following a 24-hour fast male rats (225-300 g) of the Sprague-Dawley strain were placed in a refrigerator (3°-5°C) for one hour. The adrenals were removed under pentobarbital anesthesia and analyzed for total ascorbic acid.[1] A total dose equivalent to 4 ml of cortical extract (Upjohn)† was injected subcutaneously at 3 sites. Adrenotropic hormone[2] was injected intraperitoneally in 0.9% NaCl solution.

Alterations in adrenal ascorbic acid have been used as a measure of elaboration of adrenotropic hormone by the pituitary because (1) the reduction in content of ascorbic acid in the adrenal which follows subjection of an animal to stress does not occur in the absence of the pituitary,[3] and (2) as can be seen from Table I the administration of a preparation of adrenotropic hormone, free of other pituitary activities,[2] reduces the concentration of ascorbic acid in the adrenal of the hypophysectomized rat.

After one hour in the refrigerator, the content of ascorbic acid in the adrenal of the intact rat was decreased (Table II), indicating that the pituitary was activated to increase its elaboration of adrenotropic hormone. Pretreatment with cortical extract prevented this decrease in adrenal ascorbic acid. Cortical extract does not interfere with the action of adrenotropic hormone on the adrenal cortex since administration of cortical extract did not prevent an exogenous source of adrenotropic hormone from bringing about a reduction in adrenal ascorbic acid (Table II). The dose of adrenotropic hormone selected produced a decrease in adrenal ascorbic acid equivalent to that produced by exposure to 3°-5°C for one hour and therefore approximates the amount elaborated by the pituitary of the exposed animal.

These results strongly suggest that the rate of production of adrenotropic hormone by the pituitary is regulated by the concentration of cortical hormone(s) in the blood and tissues. It may be a direct effect of the cortical hormone(s) on the anterior pituitary cells or it may be an indirect effect brought about by an increase or decrease of some regulatory substance in the tissues, the concentration of which is dependent in turn upon the level of cortical hormone(s). Ingle, Higgins, and Kendall[4] found adrenal cortical atrophy in rats after cortical hormone administration but

TABLE I.

Effect of Adrenotropic Hormone on Ascorbic Acid Content of Adrenals of Rats Three Days Following Hypophysectomy.

	No. rats	Adrenal ascorbic acid mg per 100 g fresh tissue
Hypophysectomized controls	6	326 ± 14.8*
1 hour after admin. of adrenotropic hormone (200 μg/ 100 g body wt)	5	156 ± 9.2

* Mean and standard error.

* Aided by a grant from the Utah Copper Company Research Fund.

1 Roe, J. H., and Kuether, C. A., *J. Biol. Chem.*, 1943, **147**, 399.

† We are grateful to Dr. Wilfred N. Sisk of the Upjohn Company, Kalamazoo, for a supply of this material. Alcohol was removed under reduced pressure at 50° to 60°C.

2 Sayers, G., White, A., and Long, C. N. H., *J. Biol. Chem.*, 1943, **149**, 425.

3 Sayers, G., Sayers, M. A., Liang, T. Y., and Long, C. N. H., *Endocrinology*, 1945, **37**, 96.

4 Ingle, D. J., Higgins, G. M., and Kendall, E. C., *Anat. Record*, 1938, **71**, 363.

TABLE II

	No. rats	Adrenal ascorbic acid mg per 100 g fresh tissue
Controls	18	419 ± 8.2*
1 hr after admin. of 4 ml cortical extr.	6	423 ± 8.4
1 hr at 3 to 5°C	9	314 ± 18.0
1 hr at 3 to 5°C pretreated with 4 ml cortical extr.	9	415 ± 10.1
1 hr after admin. of adrenotropic hormone (10 μg/100 g body wt)	4	314 ± 12.1
1 hr after admin. of adrenotropic hormone (10 μg/100 g body wt) pretreated with 4 ml cortical extr.	6	310 ± 7.3

* Mean and standard error.

their experiments were chronic and did not concern the physiological response to acute stress reported here.

Summary. Administration of cortical extract prevents the decrease in adrenal ascorbic acid which accompanies exposure to cold but does not prevent the decrease which results from administration of exogenous adrenotropic hormone. Since reduction in adrenal ascorbic acid is initiated by pituitary adrenotropic hormone, cortical extract must act to inhibit elaboration of this tropic hormone by the pituitary. These results have been interpreted to mean that rate of release of adrenotropic hormone from the pituitary is regulated by the concentration of adrenal cortical hormones in the blood and tissues.

15127 P

Combined Anesthesia and Hyperimmune Serum Therapy in the Treatment of Experimental Western Equine Encephalomyelitis.

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Once the symptoms of a virus disease have become evident, the virus is well established in the host cell. For this reason, it is assumed that antibodies are unable to gain access to the virus, with the result that antiserum therapy is ineffective. The close association between the virus and the host cell likewise renders chemotherapy difficult, for an agent in concentration sufficient to destroy the virus would probably injure the host cell. The ideal therapeutic agent would bring about a reversible change in the metabolism of the host cell sufficient in degree and duration to cause destruction of the virus without injuring the cell. Such an agent should have the same tissue predilection as the virus. Studies on the treatment of neurotropic virus diseases have been discouraging. The results of experiments with hyperimmune serum therapy have been contradictory, probably because of variations in the virulence and incubation period of the virus strains employed and differences in the potency of antisera.[1,2,3] Reports with chemotherapeutic agents have likewise been discouraging.[4,5] Similarly, certain antibiotics have proved ineffective in

1 Zichis, J., and Shaughnessy, H. J., *J. A. M. A.*, 1940, **115**, 633.

2 Olitsky, P. K., Schlesinger, R. W., and Morgan, I. M., *J. Exp. Med.*, 1943, **77**, 359.

3 Zichis, J., and Shaughnessy, H. J., *Am. J. Pub. Health*, 1945, **35**, 815.

4 Kramer, S. D., Geer, H. A., and Szobel, D. A., *J. Immunol.*, 1944, **49**, 273.

5 McKinstry, D. W., and Reading, E. H., *J. Franklin Inst.*, 1944, **237**, 422.

TABLE I.
Anesthesia in the Treatment of Swiss Mice Infected with Equine Encephalomyelitis Virus (Western Type).

Group	No. of mice*	% of animals dying from encephalitis during successive days after injection of virus										Survivors %
		1	2	3	4	5	6	7	8	9	10	
I. Controls	25	0	16	28 (44)†	20 (64)	8 (72)	4 (76)	4‡ (80)	0	0	0	20
II. Hyperimmune rabbit serum§	25	0	4	12 (16)	32 (48)	0	0	0	0	0	0	52
III. Ether anesthesia‖	26	0	7.7	23.1 (30.8)	7.7 (38.5)	0	0	0	0	0	0	61.5
IV. Hyperimmune rabbit serum§ and ether‖	32	0	3.1	0	0	3.1 (6.2)	0	3.1‡ (9.3)	0	0	0	90.7

* Animals which died from trauma or anesthesia are not included in this table.

† Figure in parentheses indicates cumulative percent deaths.

‡ One animal from this group sacrificed when showing symptoms suggestive of encephalitis. Virus was recovered and identified as Western equine virus by serum neutralization test.

§ Three intraperitoneal doses (0.35 cc each) of undiluted hyperimmune rabbit serum given 18 hours, 30 hours, and 42 hours subsequent to the injection of the virus.

‖ Anesthesia for 3 four-hour periods beginning 18 hours, 23 hours, and 42 hours after inoculation of virus.

experimental neurotropic virus infections.[6,7]

The influence of anesthesia on the course of several diseases affecting the central nervous system has been reported. Anesthetics, alone and in combination with specific antitoxin, decrease mortality in experimental botulism[8] and alleviate muscular spasms in tetanus.[9,10] Preliminary observations have indicated that ether anesthesia is effective in the treatment of western equine encephalomyelitis in mice.[11] Of mice treated with deep ether anesthesia soon after the intracerebral injection of virus only 58% died as compared with 92.4% of control animals. When anesthesia was delayed until the animals first showed evidence of encephalitis, 60% died as compared with 92.4% of controls. Studies are now in progress to determine the mechanism whereby anesthesia alters the course of the infection. A completed report will be published later.

In view of these observations, it seemed desirable to investigate the effectiveness of combined ether anesthesia and hyperimmune serum in the treatment of experimental western equine encephalomyelitis. The virus strain was the same as that used in the preliminary study.[11] Three- to 4-week-old white Swiss mice were used in this experiment. Each mouse was inoculated intracerebrally under light ether anesthesia with 0.03 cc of a $10^{-8.5}$ dilution of virus. All inoculated mice were separated at random into 4 groups. Group I served as controls and received no further treatment; Group II received 3 intraperitoneal doses (0.35 cc each) of undiluted hyperimmune rabbit serum 18 hours, 30 hours, and 42 hours subsequent to the injection of the virus; Group III received 3 four-hour courses of deep ether anesthesia beginning 18 hours, 23 hours, and 42 hours following injection of virus; Group IV received both hyperimmune serum as described for Group II and deep ether anesthesia as described for Group III. Pooled hyperimmune rabbit serum of high potency was used.* Animals were observed frequently for a 10-day period. Two animals

6 Parker, R. F., and Diefendorf, H. W., PROC. SOC. EXP. BIOL. AND MED., 1944, **57**, 351.

7 Sulkin, S. E., and Goth, A., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 16.

8 Bronfenbrenner, J., and Weiss, H., *J. Exp. Med.*, 1924, **49**, 273.

9 Lawen, A., *Z. f. Chir.*, 1927, **54**, 2370.

10 Kaspar, M., *Beitrag z. klin. Chir.*, 1928, **145**, 313.

11 Sulkin, S. E., Goth, A., and Zarafonetis, C., *Science*, 1945, in press.

* The results of the titration of the hyperimmune serum will be reported subsequently.

(one from Group I and the other from Group IV) showed symptoms of encephalitis on the seventh day. These animals were sacrificed and suspensions of each brain were injected intracerebrally into normal mice. In both cases virus was present in high titer and was identified as western equine encephalomyelitis virus by serum neutralization tests.

As shown in Table I, 20% of the control animals (Group I) survived the observation period of 10 days while 52% of the serum-treated animals (Group II) survived. Sixty-one and five-tenths percent of ether-treated animals survived, as compared with 90.7% of animals receiving combined ether anesthesia and hyperimmune serum (Group IV). Statistical analysis indicated that the difference in effectiveness of hyperimmune serum therapy alone as compared with that of ether anesthesia alone was not significant. The effect of combined therapy, however, is markedly greater than the effect of either method alone. Whether the serum therapy and anesthesia act independently, or whether there is a synergistic effect between the two cannot be stated from the data obtained. The possibility that anesthesia may facilitate union of virus and antibody is under investigation.

Summary. Either intraperitoneal injection of hyperimmune rabbit serum or deep ether anesthesia administered beginning 18 hours following intracerebral inoculation of virus effects a significant change in survival rate among Swiss mice infected with western equine encephalomyelitis. A combination of the two methods is more effective than either method alone.

15128

Production of Extracellular Penicillin-Inactivating Substances Associated with Penicillin Resistance in *Staphylococcus aureus.*

JOSEPH S. GOTS. (Introduced by A. B. Gutman.)

When Abraham and Chain[1] discovered the penicillin-destroying enzyme, penicillinase, the question was raised whether a bacterium's susceptibility to penicillin varied inversely with its ability to produce the enzyme. The present data do not favor complete agreement.[1-5] Too many inconsistencies exist to permit the acceptance of enzyme formation as the sole factor in explaining variation in penicillin susceptibility. In some bacteria, however, penicillin resistance may be definitely ascribed to their ability to produce penicillinase. When Kirby[6] and others[7,8] found that an active intracellular penicillin inactivator was present in resistant staphylococci and not in sensitive strains, it became apparent that perhaps in cases of varied susceptibility among various strains of the same species, the variation might be due to difference in enzyme formation. This investigation was formulated with the hope that the use of technics which are dependent on the extracellular nature of the inactivating enzyme, might throw further light on the latter concept.

Methods and Materials. The staphylococci studied were isolated primarily from cases of

1 Abraham, E. P., and Chain, E., *Nature,* 1940, **146**, 837.

2 Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W., *Lancet,* 1941, **2**, 177.

3 Bondi, A., and Dietz, C. C., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 132.

4 Bondi, A., and Dietz, C. C., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 135.

5 Woodruff, H. B., and Foster, J. W., *J. Bact.,* 1945, **49**, 7.

6 Kirby, W. M. M., *Science,* 1944, **99**, 452.

7 Hobby, G. L., and Dawson, M. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **56**, 178.

8 Manwaring, W. H., *Calif. and West. Med.,* 1944, **61**, 184.

eye infections and pyodermas. Detailed account of the latter group has been reported.[9] All of the organisms were hemolytic *Staphylococcus aureus,* positive for coagulase production and mannite fermentation. The majority of the resistant strains are known to be naturally resistant. However, in a few in which primary isolation was accomplished after penicillin therapy was started, the possibility that these had acquired resistance because of treatment, cannot be ignored. Penicillin sensitivity was determined by a tube dilution turbidimetric method.[9]

The first method employed for determining the existence of the penicillin inactivator, presumably penicillinase,* has been previously described.[10] In brief, it consists of the ability of the enzyme to diffuse from actively growing organisms into a penicillin agar medium which had been previously seeded with a very sensitive *Staphylococcus aureus.* The organisms under study are streaked on the surface of the plate, the enzyme elaborated during growth diffuses into the medium, inactivates the regional penicillin and the sensitive indicator organism is permitted to grow out as a satellitic zone of colonies around the line of streak. By measuring the width of this satellitic zone and by using a series of plates containing varying concentrations of penicillin, a fairly accurate quantitative estimation is possible. That this satellitic manifestation is actually ascribed to the inactivation of the penicillin has been shown by assaying agar plugs cut from the plate. The plugs taken from the area of satellitic growth contain no penicillin, whereas those taken from the other sections of the plate maintain original concentration.

The alternate method of penicillinase detec-

TABLE I.

Inactivation of Penicillin by *Staphylococcus aureus* on Penicillin Agar Seeded with Sensitive *Staphylococcus aureus.* Incubation: 24 hours at 37°C

Conc.of penicillin required for inhibition of organism Units per ml	Conc.of penicillin in test medium Units per ml	Total strains tested No.	Production of penicillin inactivator* (No. of strains)				
			—	+	++	+++	++++
More than 5	5.0	53	13	40			
	1.0			46	7		
	0.5			24	28	1	
	0.1				15	38	
	0.05					20	33
5.0-1.0	1.0	4	4				
	0.5		4				
	0.1		3	1			
	0.05		2	1	1		
1.0-0.5	0.5	4	4				
	0.1		4				
	0.05		4				
0.5-0.1	0.1	2	2				
	0.05		2				
0.1-0.05	0.05	7	7				

* Penicillin inactivation recorded as follows:

— = No growth of indicator organism, therefore no penicillin inactivation.

\+ = No zone of inactivation but indicator organism grew out under line of streak: slight inactivation of penicillin.

++ = Zone of inactivation 0-2 mm (measured from edge of streak).

+++ = Zone of inactivation 2-5 mm.

++++ = Zone of inactivation more than 5 mm.

[9] Waisman, M., and Gots, J. S., submitted for publication.

* No evidence is at present available that this substance is the same as the penicillinase described by Abraham and Chain.[1]

[10] Gots, J. S., *Science,* to be published.

TABLE II.
Inactivation of Penicillin by *Staphylococcus aureus* on Penicillin Agar (0.01 units per ml) Seeded with a Hemolytic *Streptococcus* (Group A). Incubation: 24 hrs at 37°C.

Conc. of penicillin required for inhibition of organism Units per ml	Total strains tested No.	Production of penicillin inactivator* (No. of strains)				
		—	+	++	+++	++++
> 5	10					7
5.0-1.0	4		1	2	1	
1.0-0.1	2		1	1		
0.1-0.05	11	11				
< 0.05	29	29				

* See Table I for explanation.

tion depends upon the ability of the enzyme to diffuse into a fluid medium. Supernatants obtained by centrifuging 24-hour broth cultures of the organisms were added in equal amounts to a penicillin dilution. The mixture was incubated for 2 hours at 37°C and residual penicillin was determined by the agar cup method using *Staphylococcus aureus,* Strain H, as the index of inhibition. By making serial dilutions of the supernatants, a quantitative estimation was possible.

Results. The results obtained by the first method are depicted in Table I. All of the highly resistant organisms, those which required more than 5 units of penicillin per ml for complete inhibition, showed a marked ability to produce a potent penicillin-inactivating substance. The amount of inactivator produced did not vary greatly. Slight production occurred in those few strains which were inhibited by penicillin concentrations of 5 to 1 units per ml, but in those which required less than 1 unit per ml for complete inhibition no production was found. Correlation with the average sensitive strains could not be accomplished because the lowest penicillin concentration possible to use in the test medium was sufficient to inhibit their growth. This difficulty was overcome by substituting for the sensitive *Staphylococcus* indicator organism a Group A hemolytic *Streptococcus.* All of the sensitive organisms could now be included in the analysis. The results with this modification are depicted in Table II. No difference could be found in the ability to produce the inactivator by the resistant organisms. Two organisms with a sensitivity range of 1 to 0.1 units per ml were added to the list of penicillinase producers. No evidence of production could be found in organisms whose sensitivity was less than 0.1 units per ml.

The results obtained with the broth culture supernatants (Table III) is in agreement with the plate method. The resistant strains produced an inactivator whereas the sensitive strains did not. It is of interest that Bondi and Dietz[3] by a similar method were unable to demonstrate inactivation of penicillin by 5 *Staphylococcus aureus* strains studied. Inasmuch as no mention was made of the degree of susceptibility of their strains, their failure may well have been due to the use of sensitive strains.

TABLE III.
Inhibition of Penicillin by Supernatants of 24-hour Broth Cultures of *Staphylococcus aureus.* A typical experiment.

Conc. of penicillin required for inhibition of organism Units per ml	Supernatant* dilution	Zone of inhibition produced† mm
> 5.0	1/2	0
	1/4	0
	1/8	0
5.0-1.0	1/2	0
	1/4	V
	1/8	V
< 1.0	1/2	27
Broth control	—	27

* Final concentration of culture supernatant contained in supernatant-penicillin mixture

† Inhibition of *Staphylococcus aureus,* Strain H, by agar cup method.

V = variable results with different strains—all were less than control.

Preliminary incubation of mixture: 2 hr at 37°C.

Discussion. In seeking an answer for the mechanism of extreme variation of penicillin

susceptibility which occurs among various strains of the same species, such as *Staphylococcus aureus,* the first logical approach is to tag the resistant members with a quality lacking in the sensitive strains. The results presented here show a marked correlation of penicillin resistance with the ability to produce a penicillin-inactivating substance, presumably penicillinase. This substance is soluble and diffuses readily into surrounding liquid and solid environment. Kirby's[6] failure to demonstrate the inactivator in Seitz filtrates of culture fluid may be due to the observation[5] that part or all of the enzyme's activity may be lost by Seitz filtration, presumably by adsorption.

In view of the available data, it cannot be denied that an organism's ability to synthesize penicillinase is not the sole factor in explaining its sensitivity or insensitivity to the action of penicillin. Sensitive organisms capable of producing penicillinase and insensitive organisms incapable of producing it have been described. Our own findings with the Shigella-Typhoid-Salmonella group of organisms confirm the findings of Bondi and Dietz.[3] This group is relatively insensitive to the action of penicillin, yet the Shigella organisms produce an inactivator and the Typhoid and Salmonella do not. Suffice it to say that the complete explanation for resistance must be awaited.

In certain bacteria at least, the resistance to penicillin can be definitely ascribed to their ability to produce penicillinase. Rather convincing are Woodruff and Foster's studies[5] on a bacillus capable of producing extracellular penicillinase at pH of 6.0 or above. At this pH the organism was uninhibited in growth by several hundred units of penicillin per ml of culture medium. When they suppressed the production of penicillinase by lowering the pH to 5.5, the organism became easily inhibited by 10 units per ml.

Those who have produced penicillin-resistant strains *in vitro* by culturing in increasing concentrations of penicillin have found that neither the resistant nor the sensitive progenitor was able to produce penicillinase. Inasmuch as we have yet to encounter a resistant strain, isolated directly from an infected process, which is incapable of producing an inactivator, it appears that there may exist a difference in the mechanism between *in vitro* acquired resistance and natural and *in vivo* acquired resistance.

Summary. A potent penicillin-inactivating substance is produced by penicillin-resistant *Staphylococcus aureus* but not by penicillin-sensitive strains. The substance is soluble and diffuses readily into both liquid and solid culture medium. The variation of susceptibility to penicillin among various strains of *Staphylococcus aureus* appears to be associated with a difference in this enzyme formation.

15129

Adaptability of Gonococcus to Four Bacteriostatic Agents, Sodium Sulfathiazole, Rivanol Lactate, Promin, and Penicillin.*†

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Chemotherapy of gonococcal infection has advanced markedly during the last decade. Sulfonamides appear to be less effective now, however, than when first introduced for the treatment of the disease. Two factors may be responsible for this evident decreased effi-

* This study was financed in part by a grant-in-aid from the Division of Venereal Diseases, U. S. Public Health Service, Washington, D.C.

† The following drugs used in this study were generously supplied by: (a) Rivanol lactate, Winthrop Chemical Company, New York, N.Y.; (b)

cacy: one, that the most susceptible strains have been eliminated leaving only the more resistant types to be transmitted throughout the population; the other, that continued exposure *in vivo* from inadequate therapy has resulted in the development of drug-fast strains.[1] Previous studies have shown that the gonococcus can be made sulfonamide-fast readily *in vitro* and recently penicillin-fast strains have likewise been developed.[2-5]

The present investigation was undertaken to find a possible solution to one of the basic problems inherent in chemotherapy; namely, acquired drug-resistance by the parasite. Because a single chemotherapeutic agent is not completely effective against an entire bacterial population, the use of several agents of widely-divergent chemical structures was an obvious approach to the problem. Such a procedure should insure a greater degree of inhibition of metabolic activity of the bacterial cell. Thus drug-resistance becomes more remote. To test the validity of this hypothesis, observations were made on the anti-bacterial effect of the combination of 4 therapeutic agents on the gonococcus. For comparison, the effects of the same drugs used individually were also determined. Information on the concurrent use of several agents will aid in solving the problem of drug-resistance. Furthermore, the combined administration of several agents for a specific parasite offers clinical possibilities.

Technic. The bactericidal agents selected for the study were sodium sulfathiazole, rivanol lactate, promin, and penicillin, representing respectively a sulfonamide, an acridine derivative, a sulfone, and an antibiotic agent. The available information on the mode of action of each substance indicates a special action for each that is consonant with the individuality of its chemical configuration and which has formed the basis for their selection in this study. Five non-resistant strains of the gonococcus identified as No. 515, 3215, 5609, 5723, and 8071, were selected for the experiment.‡ Two of the strains were isolated from male patients with urethritis and 3 from patients with cervicitis. The basic culture medium in which the organisms were grown was Douglas's broth with 0.05% KNO_3, 0.04% KH_2PO_4, and 5% lapine blood.

The 5 strains of the organism were grown in the above medium with gradually increasing concentrations of each agent separately. Initial concentrations used were 0.12 mg% of sodium sulfathiazole and of rivanol lactate (*i.e.*, 0.12 mg per 100 ml of medium), 32 mg% of promin, and 1.0 units % of penicillin (*i.e.*, 1 Oxford unit per 100 ml of medium). The strains were likewise subjected to the combined action of the following 3 drugs: sodium sulfathiazole, rivanol lactate, and promin, as well as to the same 3 combined with penicillin. Preliminary tests had shown that when the drugs were used in combination, the inhibitory action of each was approximately additive. The concentrations of the combined agents in which the cultures were first introduced, therefore, were approximately one-fourth that of the initial concentrations of the individual drugs, namely, 0.03 mg% rivanol lactate and sodium sulfathiazole, 8 mg% promin, and 0.25 unit % penicillin. Similarly, when 3 drugs were used, the concentration of each was one-third that initially permitting growth.

All cultures were transferred to the same concentration of drug in which they were growing and to a greater concentration every 48 hours for 4 months. Subcultures for viability were made on "chocolate" agar at the same time but on alternate days.

Results. The degree of resistance to each

Promin, Parke, Davis and Company, Detroit, Mich.; (c) Penicillin, Committee on Medical Research of the Office of Scientific Research and Development, Washington, D.C.

1 Carpenter, C. M., Ackerman, H., Winchester, M. E., and Whittle, J., *Am. J. P. H.*, 1944, **34**, 250.

2 Boak, R. A., Charles, R. L., and Carpenter, C. M., *Am. A. Advancement Sc.*, Publ. No. 11, 118.

3 Westphal, L., Charles, R. L., and Carpenter, C. M., *Ven. Dis. Inform.*, 1940, **21**, 183.

4 Kirby, W. M. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 175.

5 Bahn, J. M., Ackerman, H., and Carpenter, C. M., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 21.

‡ Hereinafter multiple concentrations of each drug are given in numerical order of the 5 strains of the gonococcus studied, *i.e.*, No. 515, No. 3215, No. 5609, No. 5723, and No. 8071.

TABLE I.
Resistance of 5 Strains of the Gonococcus to 4 Drugs, Individually and in Combination.

Strain No.	Sodium Sulfathiazole Concentration		Rivanol lactate Conc.		Promin Conc.		Penicillin Conc.		Sodium sulfathiazole	Rivanol lactate	Promin	Penicillin
	Initial*	Maximal†	Initial	Max.	Initial	Max.	Initial	Max.	Fold increase in conc.			
	mg%‡		mg%		mg%		units %§					
					Drugs individually							
515	.05	64.00	.12	8.00	32	256	0.50	100	1280	67	8	200
3215	.12	128.00	.12	8.00	32	512	1.00	100	1067	67	16	100
5609	.12	128.00	.12	8.00	32	256	0.50	200	1067	67	8	400
5723	.12	16.00	.12	16.00	32	256	0.50	100	133	133	8	200
8071	.12	2.00	.12	8.00	32	128	1.00	8	17	67	4	8
					Three drugs in combination							
515	.04	.32	.04	.32	10	80	—	—			8	
3215	.04	.64	.04	.64	10	160	—	—			16	
5609	.04	.32	.04	.32	10	80	—	—			8	
5723	.04	.48	.04	.48	10	120	—	—			12	
8071	.04	.32	.04	.32	10	80	—	—			8	
					Four drugs in combination							
515	.03	.03	.03	.03	8	8	.25	.25			0	
3215	.03	.03	.03	.03	8	8	.25	.25			0	
5609	.03	.03	.03	.03	8	8	.25	.25			0	
5723	.03	.03	.03	.03	8	8	.25	.25			0	
8071	.03	.03	.03	.03	8	8	.25	.25			0	

* Concentration initially permitting growth.
† Maximal concentration to which growth was adapted.
‡ Milligrams of drug per 100 ml medium.
§ Oxford units of penicillin per 100 ml medium.

drug acquired after 4 months' exposure varied greatly with the individual strains of the gonococcus. The maximal concentration of each drug tolerated by the strains, No. 515, 3215, 5609, 5723, and 8071, at the end of that period was as follows: sodium sulfathiazole 1280, 1067, 1067, 133, and 17 times greater, respectively, than those which initially permitted growth, and in rivanol lactate 67, 67, 67, 133, and 67; promin 8, 16, 8, 8, and 4, and penicillin 200, 100, 400, 200, and 8 times greater than the original concentration.

The 5 strains of the gonococcus acquired only a slight degree of fastness after 4 months' exposure to sodium sulfathiazole, rivanol lactate, and promin when used concurrently. The maximal concentrations in which these strains grew were 8, 16, 8, 12, and 8 times greater than the amounts of the mixed compounds into which the 5 parent strains were first introduced. When penicillin was added to the mixture of the same 3 drugs, none of the strains acquired resistance to the combined effects (Table I).

Discussion. The mechanism by means of which a bacterial cell becomes resistant to gradually increasing concentrations of bactericidal agents is complex. Knowledge of the metabolism of the cell and especially an understanding of its enzymic systems are necessary. The present studies serve as an indirect approach to the problem. The magnitude of increase ("fold increase") of the initial concentration of each agent permitting growth of the strain, served as the basis for comparison of acquired resistance to the drugs and undoubtedly constituted a sufficiently sensitive index. Fastness to each drug developed at different rates and to various degrees. This suggests that the mechanism by which the gonococcus becomes fast to an agent varies with the nature of the compound to which the cells were exposed. For instance, the adaptation to growth in the presence of a sulfonamide requires an altered cell metabolism probably different from that permitting growth in the presence of a sulfone, an acridine derivative, or an antibiotic agent.

Our observations indicate that promin and penicillin were more toxic for the gonococcus than sodium sulfathiazole and rivanol lactate and that the organisms could not adapt their growth readily to the presence of the former two substances. Penicillin, however, appeared to play the most important role in inhibiting the acquisition of resistance because its addition to the other compounds prevented growth of each strain tested in concentrations greater than those initially permitting growth. Since the cells were unable to survive *in vitro* in the presence of all 4 agents to which resistance had been acquired individually, a possible clinical application is indicated.

Summary. Five strains of the gonococcus were adapted to grow in gradually increased concentrations of each of the following agents: sodium sulfathiazole, rivanol lactate, promin, and penicillin. Only slight adaptation to growth was attained, however, when the strains were exposed to gradually increased concentrations of sodium sulfathiazole, rivanol lactate, and promin in combination. When penicillin was added to the above 3 drugs, none of the 5 strains developed resistance to their combined effects. Interpretation of the results and the possible clinical application of the *in vitro* observations are presented.

INDEX

Preliminary articles are indicated by the letter P after the number of the article. All others are non-preliminary, *i. e.*, complete.

ALLINSON, M. J. C. — Colorimetric Estimation of Penicillin in Aqueous Solutions ... 293

ARNOW, L. E., 196.

ARTOM, C., FISHMAN, W. H., and MOREHEAD, R. P. — The Relative Toxicity of *l*- and *dl*- Serine in Rats ... 284

ARTOM, C., 288.

BANG, F. B., 257.

BLOOMFIELD, SYLVAN — Parasympathomimetic Effect of Aqueous Humor in Human Eyes With and Without Chronic Simple Glaucoma ... 293

BRUNDAGE, R., 301.

BUTZ, L. W., and FRAPS, R. M. — No Androgen in Corn Pollen ... 213

CARLQUIST, J. H., 298.

CHARGAFF, E., 245.

CLAPPER, W. E., and CARLQUIST, J. H. — Brain Abscess and Meningitis Caused by a Diphtheroid Resembling *Corynebacterium ulcerogenes* ... 298

CLARK, P. F., 279.

DARBY, W. J., and JONES, E. — Treatment of Sprue with Synthetic *L. casei* Factor (Folic Acid, Vitamin M) ... 259

DOMINGUEZ, R., and POMERENE, E. — Calculation of the Rate of Absorption of Exogenous Creatinine ... 173

DRAGER, G. A., 200.

EATON, M. D. — Serological Differentiation of Primary Atypical Pneumonia from Virus Pneumonia of the Psittacosis Group ... 231

ELVEHJEM, C. A., 279.

FANTA, P. E., 228.

FERNANDEZ, E. B., 200.

FISHBERG, E. H., and VORZIMER, J. — Effect of Pyridoxine on Granulopenia Caused by Thiouracil ... 181

FISHMAN, W. H., and ARTOM, C. — Variations of Some Constituents in the Urine of Rats Receiving *dl*-Serine and *dl*-Alanine 288

FISHMAN, W. H., 284.

FOLEY, J. O. — Sensory and Motor Axons of the Chorda Tympani ... 262

FRAPS, R. M., 213.

GELLHORN, E., 247.

HAIRSTON, N. G., 257.

HANGER, F. M., 245.

HANSEN, R., 272.

HENNY, G. C., 237.

HEYMANN, W., and SEKERAK, B. — "Renal Hyperlipemia" in Dogs and Rats ... 276

HIGASHI, A., 196.

HODES, H. L., THOMAS, L., and PECK, J. L. — Cause of an Outbreak of Encephalitis Established by Means of Complement-Fixation Tests ... 220

HODGE, H. C., 228.

HOGAN, A. G., 211.

JONES, E., 259.

KASS, E. H., and WAISBREN, B. A. — A Method for Consistent Induction of Chronic Hyperglycemia with Alloxan ... 303

KAZAL, L. A., HIGASHI, A., and ARNOW, L. E. — Horse Brain Thromboplastin. Effect of Various Factors on Activity of Horse Brain Extracts ... 196

KENDALL, F. E., MEYER, W., LEWIS, L., and VICTOR, J. — Alloxan Diabetes in Rabbits. Production of Hypercholesterolemia, Hyperlipemia, and Adrenal Cortical Lesions ... 190

KENNEY, M. — Experimental Intestinal Myiasis in Man ... 235

KLINE, B. E., and RUSCH, H. P. — Effects of Prolonged Irradiation of Mice with Fluorescent Light ... 254

KOETS, P., 239.

LAQUEUR, G. L., and KOETS, P. — Growth of Corpus Luteum in Rats During Pregnancy and Following Injections of Testosterone ... 239

LEATHEM, J. H. — Plasma Protein Concentrations in Adrenalectomized Rats Maintained on Sodium Chloride 260

LEWIS, L., 190.

LICHSTEIN, H. C., WAISMAN, H. A., McCALL, K. B., ELVEHJEM, C. A., and CLARK, P. F. — Influence of Pyridoxine, Inositol, and Biotin on Susceptibility of Swiss Mice to Experimental Poliomyelitis ... 279

McCALL, K. B., 279.

McCLOSKEY, C. M., and PORTER, J. R. — Utilization of Certain Rare Sugars by Microorganisms ... 269

MACHT, D. I. — Influence of Sulfonamides and Para-Aminobenzoic Acid on the Growth of *Lupinus albus* 217

MEYER, W., 190.

MILLER, I., 272.

MOREHEAD, R. P., 284.

MUHRER, M. E., and HOGAN, A. G. — Effect of Thiouracil on Growing Swine ... 211

ORTEN, J. M., 301.

PECK, J. L., 220.

POMERENE, E., 173.

PORTER, J. R., 269.

POTH, E. J., FERNANDEZ, E. B., and DRAGER, G. A. — Prevention of the Formation of End-Bulb Neuromata ... 200

RECANT, L., CHARGAFF, E., and HANGER, F. M. — Comparison of the Cephalin-Cholesterol Flocculation with the Thymol Turbidity Test ... 245

RICHARDSON, A. P., WALKER, H. A., MILLER, I., and HANSEN, R. — Metabolism of Methyl and Benzyl Esters of Penicillin by Different Species ... 272

RUSCH, H. P., 254.

SAFFORD, H., and GELLHORN, E. — Blood Sugar Level and Autonomic Balance ... 247

SCHARRER, B. — Experimental Tumors After Nerve Section in an Insect ... 184

SCHILLER, J. — Vaginal Response to Adrenal Stimulation ... 207

SEKERAK, B., 276.

SMITH, A. H., 301.

SMYTH, C. J., BRUNDAGE, R., ORTEN, J. M., and SMITH, A. H. — Fumaric Acid Salts as Hydrogogue Cathartics 301

SPIEGEL, E. A., HENNY, G. C., WYCIS, H. T., and SPIEGEL-ADOLPH, M. — Impedance Changes in the Cerebrum Following Concussion ... 237

SPIEGEL-ADOLF, M., 237.

STEENKEN, W., JR., and WAGLEY, P. F. — Comparison of the Golden Hamster to the Guinea Pig Following Inoculations of Virulent Tubercle Bacilli ... 255

SURE, B. — Dietary Requirements for Fertility and Lactation. Rice and Its By-Products as Sources of Pyridoxine and Pantothenic Acid for Reproduction and Lactation ... 250

THOMAS, L., 220.

TORREGROSA, M. V. — Incidence of the Eight Rh Types Among 179 White Puerto Ricans ... 215

TOWBIN, E. J., FANTA, P. E., and HODGE, H. C. — Porphyrin of Harder's Gland ... 228

TRAGER, W., BANG, F. B., and HAIRSTON, N. G. — Relation of Plasma Level of Atabrine to Morphology and Motility of *Plasmodium Vivax* ... 257

VICTOR, J., 190.

VISSCHER, F. E. — Renal Clearance of β-Hydroxybutyric Acid in Dog ... 296

VORZIMER, J., 181.

WAGLEY, P. F., 255.

WAISBREN, B. A., 303.

WAISMAN, H. A., 279.

WALKER, H. A., 272.

WOOLLEY, D. W. — Observations on Antimicrobial Action of 2,3-Dichloro-1,4-Naphthoquinone, and Its Reversal by Vitamins K ... 225

WYCIS, H. T., 237.

YUDKIN, W. H. — Occurrence of Thiaminase in Marine Teleosts 268